THE IMMUNOBIOLOGY OF HLA-HAPLOIDENTICAL HEMATOPOIETIC CELL TRANSPLANTATION

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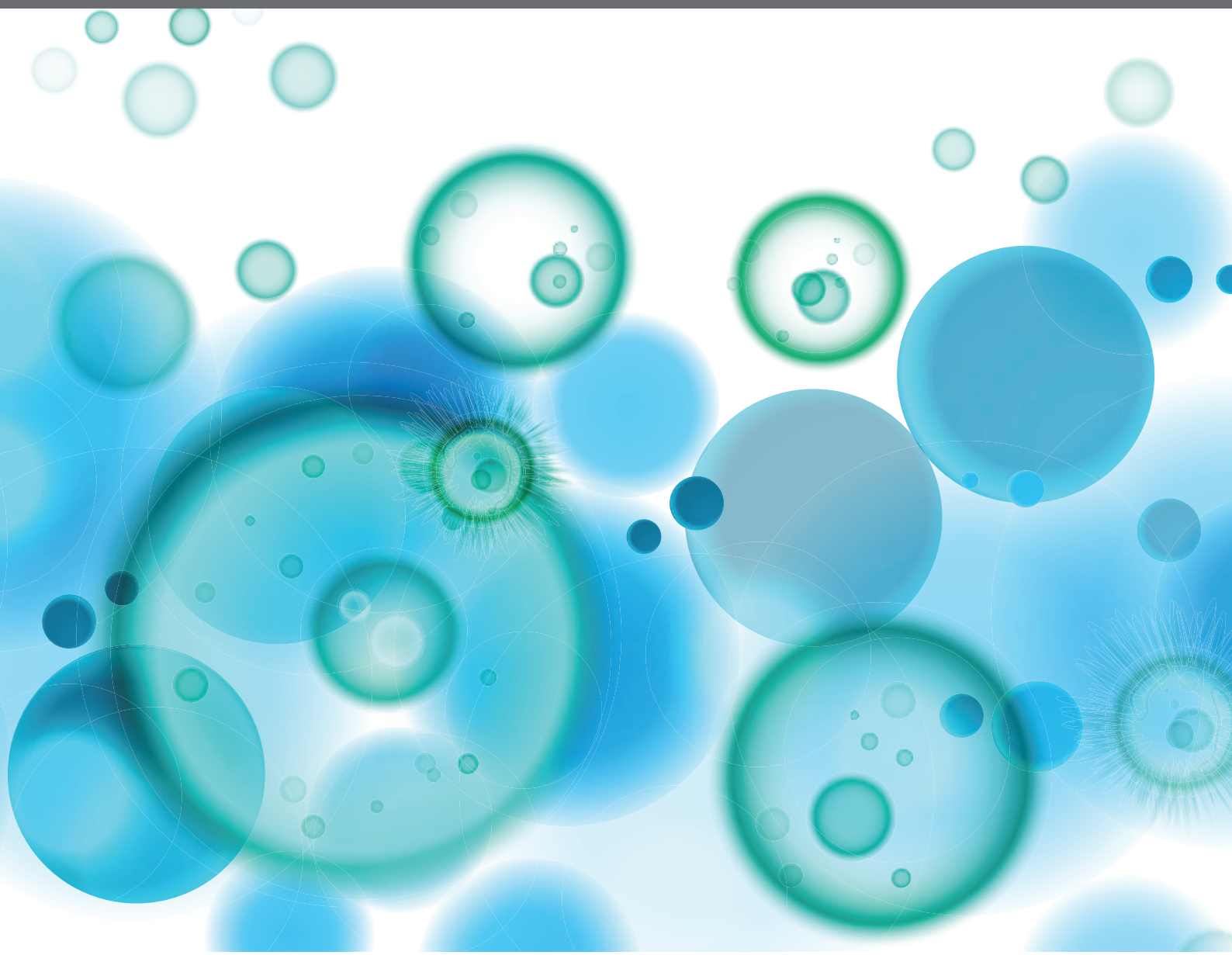

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THE IMMUNOBIOLOGY OF HLA-HAPLOIDENTICAL HEMATOPOIETIC CELL TRANSPLANTATION

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EDITORIAL
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Editorial: The Immunobiology of HLA-Haploidentical Hematopoietic Cell Transplantation

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Keywords: haploidentical transplantation, T-cell depleted grafts, T-cell-replete grafts, post-transplantation cyclophosphamide, immune reconstitution, adoptive cell therapy, regulatory T cells, immune evasion

Editorial on the Research Topic

The Immunobiology of HLA-Haploidentical Hematopoietic Cell Transplantation

Allogeneic hematopoietic cell transplantation from a related donor who is mismatched for one human leukocyte antigen (HLA) haplotype [HLA-haploidentical transplantation (haploHCT)] has become an effective treatment for patients with high-risk hematologic malignancies, the majority of whom lack an HLA-matched donor. Bi-directional responses to mismatched HLA molecules are associated with high risk of adverse immune reactions driven either by donor alloreactive T cells against recipient tissues [graft-vs.-host disease (GvHD)], or by host alloreactive T cells against the graft (graft rejection). At the same time, donor alloreactive T cells exert a strong graft-vs.-tumor effect because of the mismatch within the unshared HLA haplotype, and this contributes to relapse prevention. The present Research Topic describes how immunological studies pave the way for the development of successful protocols for haploHCT and continuous advancements in the field.

The first safe and effective haploHCT protocol consisted of a myeloablative and immunosuppressive conditioning regimen, the infusion of a "mega-dose" of T-cell-depleted hematopoietic stem cells harvested initially by sedimentation and then through a $CD34^+$ positive selection, and no post-transplant immunosuppression. This approach achieved a high rate of engraftment and low incidence of GvHD. The strong graft-vs.-leukemia effect mediated by alloreactive NK cells was unveiled in this setting. However, infection-related mortality was high because donor post-transplant immune reconstitution was slow. Aversa et al. review how T-cell depleted haploHCT has evolved in order to improve outcomes. One approach is to selectively deplete the graft from T-cell subpopulations that can be alloreactive, such as $\alpha\beta^+$ T cells or $CD45RA^+$ naïve T cells. Other groups have developed adoptive cell therapies that aim at boosting immunity against pathogens and malignant cells while controlling adverse alloreactions. Zhang and Tey focus on adoptive T-cell therapies that can provide pathogen- or tumor-specific immunity, or broad T-cell immunity. Among them, switch gene-modified T cells are transduced with suicide genes (e.g., HSV-derived thymidine kinase or inducible caspase 9 genes) and can be conditionally deleted when GvHD occurs. More recently, the importance of the role of $CD4^+Foxp3^+$ regulatory T cells in inducing tolerance after allogeneic HCT has been appreciated. Mancusi et al. report how adoptive immunotherapy with donor conventional T cells under the control of regulatory T cells prevents GvHD, improves immune reconstitution, and preserves the graft-vs.-leukemia effect after haploHCT in the absence of post-transplant immunosuppression.

A strong innovation in the field has been the development of feasible and effective protocols of unmanipulated (T-cell-replete) haploHCT, based on novel strategies that control T-cell

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alloreactivity and induce T-cell tolerance. One main protocol of T-cell-replete haploHCT is based on the modulation of T-cell immunity using granulocyte colony-stimulating factor-primed grafts, anti-thymocyte globulin, and intensive post-transplant immunosuppression. Chang et al. report further improvements of this protocol, that include the definition of parameters to identify patients with higher risk of post-transplant complications, interventions to overcome poor graft function, and the prophylaxis and treatment of viral infections and relapse.

The second main approach to T-cell-replete haploHCT is the administration of high-dose cyclophosphamide following graft infusion [post-transplantation cyclophosphamide (PTCy)], which allows for the immunomodulation of alloresponses while sparing donor hematopoietic stem cells. Current trials of haploHCT with PTCy are associated with high engraftment rates and low incidences of severe acute and chronic GvHD as well as non-relapse mortality. This approach has become widely adopted over the last several years, but the mechanism by which PTCy prevents GvHD remain incompletely understood. Kato et al. describe the work performed in the 1980s and early 1990s in murine skin allografting models that was instrumental in developing PTCy clinically and established the first model of understanding for how PTCy may work to control alloreactive responses. An adaptation of this model has since become widely accepted in the field. Nunes and Kanakry describe limitations of this model and recent work that challenges this model and provides the framework for a new model of understanding of the mechanisms by which PTCy prevents GvHD. Williams et al. describe the successful and increasingly widespread application of the PTCy approach to HLA-matched related or unrelated donor transplantation platforms, building on the success in haploHCT.

The above protocols of haploHCT variously affect the reconstitution of functional immune cell subsets after transplant, which impacts on outcomes. Zaghi et al. highlight the role of innate lymphocytes, such as NK cells, innate lymphoid cells, and γδ T cells, which tend to reconstitute faster than adaptive B and T cells and could provide early protection against infections and relapse. However, results in patients with unfavorable prognosis, such as those with chemo-resistant disease, are still unsatisfactory, and relapse is still a major cause of treatment failure. Rovatti et al. discuss the mechanisms of immune evasion in allogeneic HCT, such as genomic loss or downregulation of HLA molecules, up-regulation of T-cell inhibitory ligands, release of mediators of T-cell exhaustion, and inhibition of the release of pro-inflammatory cytokines. Among them, the genomic loss of the mismatched HLA haplotype expressed by leukemic cells (known as "HLA loss") has been detected in ∼30% of relapses after haploHCT compared with 5–10% in unrelated donor HCT, possibly reflecting the strong pressure exerted by donor T cells on the mismatched HLA haplotype. Baumeister et al. provide an exhaustive overview of the current protocols for haploHCT, the post-transplant reconstitution of immune cell subpopulations, and the perspectives to further improve outcomes.

The present Research Topic reports how haploHCT has become an effective and widespread treatment so much that survival rates are similar to those after HLA-matched sibling or unrelated donor HCT. Moreover, haploHCT is a feasible platform for further improvements, including the combination with innovative immunotherapies such as bi-specific antibodies and CAR-T cells and CAR-NK cells.

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REVIEW
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Adoptive T Cell Therapy Following Haploidentical Hematopoietic Stem Cell Transplantation

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Delayed immune reconstitution and the consequently high rates of leukemia relapse and infectious complications are the main limitations of haploidentical hematopoietic stem cell transplantation. Donor T cell addback can accelerate immune reconstitution but the therapeutic window between graft-vs.-host disease and protective immunity is very narrow in the haploidentical transplant setting. Hence, strategies to improve the safety and efficacy of adoptive T cell transfer are particularly relevant in this setting. Adoptive T cell transfer strategies in haploidentical transplantation include the use of antigen-specific T cells, allodepletion and alloanergy induction, immune modulation by the co-infusion of regulatory cell populations, and the use of safety switch gene-modified T cells. Whilst common principles apply, there are features that are unique to haploidentical transplantation, where HLA-mismatching directly impacts on immune reconstitution, and shared vs. non-shared HLA-allele can be an important consideration in antigen-specific T cell therapy. This review will also present an update on safety switch gene-modified T cells, which can be conditionally deleted in the event of severe graft- vs.-host disease or other adverse events. Herpes Virus Simplex Thymidine Kinase (HSVtk) and inducible caspase-9 (iCasp9) are safety switches that have undergone multicenter studies in haploidentical transplantation with encouraging results. These gene-modified cells, which are trackable long-term, have also provided important insights on the fate of adoptively transferred T cells. In this review, we will discuss the biology of post-transplant T cell immune reconstitution and the impact of HLA-mismatching, and the different cellular therapy strategies that can help accelerate T cell immune reconstitution after haploidentical transplantation.

Keywords: haploidentical transplant, T cell immune reconstitution, adoptive T cell therapy, antigen-specific T cells, safety switch, gene modification

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INTRODUCTION

The past decade has seen a sharp increase in the number of haploidentical hematopoietic stem cell transplants (HSCT), which is driven by smaller family sizes and increased transplant activity amongst patients of non-European ancestries that are not well-represented in volunteer donor registries (1, 2). At the same time, outcomes of haploidentical HSCT have steadily improved,

with some specialized centers reporting outcomes that are comparable to those of matched sibling and matched unrelated transplants (3–8). This remarkable progress can be attributed to advances in graft-engineering and critical refinements in conditioning regimen and immunosuppressive regimen, which together overcome the key barriers of graft rejection and lethal graft- vs.-host disease (GVHD). Three major haploidentical HSCT approaches have emerged: (1) intensive myeloablative conditioning regimen combined with *in vivo* T cell depletion with anti-thymocyte globulin (ATG) to enable the engraftment of megadose CD34-selected T cell depleted graft, which was pioneered in Perugia, Italy (9); (2) non-myeloablative or reduced-intensity conditioning followed by the infusion of unmanipulated T cell replete bone marrow or peripheral blood stem cell graft, followed by the depletion of alloreactive T cells *in vivo* with high-dose post-transplant cyclophosphamide (PTCy), which was pioneered in Baltimore, USA (10); and (3) high-intensity myeloablative conditioning regimen that incorporates ATG-based *in vivo* T cell depletion and intensive immunosuppression followed by the infusion of granulocyte colony-stimulating factor (G-CSF)-primed bone marrow or peripheral blood stem cell grafts, which was pioneered in Beijing, China (11). Despite the promising outcomes, infectious complications and relapse of underlying malignancies remain significant sources of transplant failure, especially following *ex vivo* T cell deplete haploidentical HSCT, where T cell immune reconstitution is particularly delayed. T cell reconstitution is numerically more rapid after T cell replete haploidentical HSCT using either PTCy or the Beijing approach (12–14), but the qualitative immune dysfunction that characterizes all forms of allogeneic HSCT is exacerbated by HLA-disparity in the haploidentical setting.

Adoptive T cell transfer has an established role in allogeneic HSCT and are particularly relevant in the haploidentical setting, where immune reconstitution is poorer and the immediate and near-universal availability of related donors provide added opportunities for advanced graft engineering and cellular therapy. The principles of adoptive T cell transfer after HLA-matched transplantation is broadly applicable to other transplant settings but the risk of GVHD, at least from donor-derived T cell therapy, is higher in the presence of HLA-mismatch, especially in haploidentical HSCT, where the precursor frequency of alloreactive T cells can be orders of magnitude higher (15). This lower therapeutic index has inspired new approaches, including the use of safety-switch modified T cells that can be conditionally deleted in the event of severe GVHD (16), and immune-modulatory approaches, such as the co-infusion of regulatory T cells (Tregs) together with conventional T cells (Tcons) (17), and allospecific T cell depletion and anergy induction (18).

In this manuscript, we will briefly review the features of immune reconstitution after haploidentical HCST, followed by detailed discussions on the use of adoptive T cell transfer, including an update on safety-switch gene-modified T cell addback.

T CELL RECONSTITUTION FOLLOWING HAPLOIDENTICAL HSCT

The pattern and tempo of immune reconstitution is influenced by the specific transplant technique. In all cases, innate immunity reconstitutes faster, with natural killer (NK) cells and γδ-T cell reaching normal numbers within the first few weeks post-transplant (19). The reconstitution of adaptive immunity, both cellular and humoral, is significantly slower (20). T cells, which are key mediators of both GVHD and graft- vs.-leukemia effect, reconstitute via two distinct pathways: the expansion of T cells that are contained within the stem cell graft, and the development of new thymic emigrants from donor hematopoietic stem cells (20, 21). The lymphopenic environment created by pre-transplant conditioning promotes cytokine-driven expansion of T cells within the graft. Subsequent antigen exposure, including viral antigens, provides further expansion of antigen-specific T cells (14, 22). In T cell deplete transplants where there are only small numbers of contaminating T cells, these early reconstituting T cells have a narrow T cell receptor (TCR) repertoire. In one study, 80% of the T cells at 2 months post-transplant could be accounted by as few as 13–504 TCR clonotypes, with overlaps found with T cells in the graft (23). In haploidentical HSCT with PTCy, the number of T cells infused is large, but a significant proportion is subsequently deleted by cyclophosphamide. Although T cell count recovery is much more rapid, the T cells are predominantly CD45RA(-)CCR7(-) effector memory and CD45(+)CCR7(-) terminally differentiated TEMRA in phenotype, and have a lower TCR repertoire diversity that is not fully restored even at 1 year post-transplant (14). The impact of transplantation platform on TCR repertoire reconstitution is difficult to quantify because of differences in baseline patient characteristics and TCR sequencing technologies, but delayed reconstitution of TCR repertoire is common to all allogeneic HSCT, including fully HLA-matched transplantation (24), and can have important implications on functional immune reconstitution.

Restoration of TCR repertoire through the export of naïve T cells from the thymus is a slow process which takes 1–2 years and is affected by recipient age and GVHD (25–27). Thymic T cell selection is determined by the affinity of TCR to peptide-MHC expressed in the thymic microenvironment, specifically by the thymus epithelial cells (TECs) and bone-marrow-derived antigen-presenting cells (28). T cell selection comprises two sequential stages: positive selection which prevents death by neglect of double positive thymocytes that express TCR of intermediate affinity for self-peptide MHC on cortical TECs; followed by negative selection, during which thymocytes with high-affinity to self-peptide MHC are deleted by apoptosis. Negative selection occurs predominantly in the thymic medulla and is mediated by a broader range of cell types, including medullary TECs and a range of bone marrow-derived cells: resident and migratory conventional dendritic cells, plasmacytoid dendritic cells and B cells (28, 29). Following allogeneic HSCT, the hematopoietic antigen presenting cells are of donor origin, whereas the TECs remain of recipient origin.

In haploidentical HSCT, there is a degree of mismatch between the MHC that effect thymic selection and the MHC that is expressed on peripheral tissue. In addition, the thymus is a target organ for GVHD, and the thymic stroma can also be damaged by the conditioning regimen. The resulting thymic dysfunction impairs thymic export of naïve T cells and disruption to negative selection allows escape of autoreactive T cells to the periphery, which exacerbates GVHD and leads to further thymic dysfunction (30, 31). The recovery of thymic export after allogeneic HSCT also disproportionately affects Tregs, with lower proportions of recent thymic emigrants within the Treg compartment as compared to CD4 and CD8 conventional T cell compartments, and this imbalance further contributes to post-transplant immune dysregulation (32).

Cytomegalovirus (CMV) is a strong driver of early T cell reconstitution, especially in the CD8 effector memory and TEMRA compartments (14, 22). Interestingly, CMV also promotes a convergence of TCR repertoire between recipients and donors, likely because CMV-specific T cells can constitute a significant fraction of the T cell compartment (14). It is important to note here that HLA-mismatching in itself has an impact on functional immune reconstitution: a proportion of pathogen-specific memory T cells transferred within the graft will bear TCRs that are restricted to non-shared HLA alleles, which are not expressed by pathogen-infected recipient non-hematopoietic cells. Conversely, there is a deficiency of T cells that can bind recipient HLA alleles that are not present in the donor. The net effect is a functional defect in the TCR repertoire, which will take months and years to recover, with the reconstitution of new thymic emigrants.

HIGH FREQUENCY OF MHC-ALLOREACTIVE T CELLS AS A LIMITATION TO UNMANIPULATED T CELL ADDBACK

The delayed addback of defined doses of T cells after T cell deplete transplantation can potentially accelerate T cell reconstitution without excessive risks of life-threatening GVHD. Intensive pre-transplant conditioning induces tissue damage, and the resulting cytokine storm activates recipient antigen-presenting cells and enhances the priming and Th1/Th17 polarization of donor alloreactive T cells (33, 34). Hence, donor T cell infusion after the resolution of cytokine storm should in theory be associated with lower risks of severe GVHD. However, in the presence of HLA-disparity, the risk of GVHD is high even with small T cell doses. In early studies using T cell depletion by soybean agglutination and E-rosetting, fatal acute GVHD occurred with T cell graft contamination of 1×10^6/kg despite concurrent administration of ATG (35). Indeed, the actual safe dose of T cell would turn out to be much lower.

The precursor frequency of alloreactive T cells in HLA-mismatched transplantation is estimated at 1–10% based on *in vitro* and *in vivo* functional assays (15, 36). The molecular basis for this vast repertoire of MHC-alloreactive T cells is not fully elucidated. Current evidence suggests that TCRs that are positively selected on low and intermediate affinity interactions with self MHC/peptide can sometimes cross-react with allogeneic MHC/peptide because there is a degree of flexibility in TCR-MHC/peptide interaction. These allogeneic MHC/peptide interactions can be of high affinity because the mismatched recipient MHC is not expressed in the donor's thymus and, hence, physiological deletion of high affinity TCR has not occurred (15). Cross-reactivity can occur by molecular mimicry: for example, a single TCR that is specific to HLA-B*0801 presenting FLRGRAYGL peptide from an Epstein-Barr virus (EBV) nuclear antigen can also recognize HLA-B*3501 presenting KPIVVLHGY peptide from human cytochrome P450 because of structural homology in the regions implicated in TCR recognition "hot spots" (37, 38). In other cases, cross-reactivity occurs without a need for molecular mimicry: a single TCR can recognize self- and allogeneic-MHC/peptide complexes through unique amino acid contacts, which results in divergent binding orientations (36). The relative contribution of the different molecular mechanisms is yet to be defined but it is clear that the magnitude of allogeneic MHC cross-reactive T cells can be very large: in one study, 45% of virus-specific T cell clones were found to cross-react with allogeneic HLA molecules *in vitro* (39). Thus, even small doses of T cells has the potential to cause life-threatening GVHD in haploidentical transplantation.

G-CSF priming is perhaps one of the earliest, albeit unintended, form of T cell immune modulation. The incidence of acute GVHD after transplantation with G-CSF mobilized peripheral blood stem cell grafts is comparable to that after bone marrow grafts despite the former having 10-fold higher number of T cells (40). This has been attributed to Th2 polarization (41), promotion of regulatory T cells (42) and expansion of regulatory antigen-presenting cells (43), which collectively contribute to the lower rate of acute GVHD, although at the expense of increased Th17 polarization and chronic GVHD (44, 45). Despite this, the safe dose of G-CSF-primed donor lymphocyte infusion (DLI) is very low. In a dose-finding study, adult patients undergoing CD34-selected haploidentical HSCT without post-transplant immunosuppression received prophylactic DLI from day +28 onwards, using the CD34-negative fraction of the graft. A dose of 3×10^4 $CD3^+$ T cells/kg induced grade II acute GVHD in 2 out of 2 patients. Three lots of monthly DLI at 1×10^4 CD3/kg/dose was found to be safe but incremental DLI to 3×10^4 $CD3^+$ T cells/kg again induced high rates of acute GVHD (**Table 1**) (46). CD4 immune reconstitution remained very delayed despite DLI, relapse rate was high in patients without GVHD, and two patients in this small study later died from GVHD. A similar approach was studied in the pediatric population with G-CSF-primed DLI at 4–6 weeks after T cell deplete transplant. In this study, weekly methotrexate was administered post-DLI to limit the risk of acute GVHD. It was found that modest T cell doses at $3–5 \times 10^4$ cells/kg could achieve their target endpoint of at least 67% of children reaching CD4 T cell count >100/uL by day +120 post-transplant, nearly all of which were memory T cells, suggesting that they originated form the DLI fraction. Grade II–IV acute GVHD occurred in seven out of 35 children (49). In the T cell replete setting, investigators at Beijing used cryopreserved excess peripheral blood stem cell grafts for subsequent DLI, either

in response to disease relapse or prophylactically in high-risk patients. In one study, 20 patients with leukemia relapse received G-CSF primed DLI at a dose of 0.07–4.4 $\times$ 10^8 $CD3^+$ T cells/kg. Grade III-IV acute GVHD occurred in 5 out of 9 patients who did not receive GVHD prophylaxis and in 1 out of 11 patients who received post-DLI cyclosporine or methotrexate; with overall disease response rate of 70% (47). In a recent study from the same group, 31 patients who underwent haploidentical HSCT for high-risk leukemia received prophylactic DLI at a median of 77 days post-transplant, at a median dose of 1.8 $\times$ 10^7 $CD3^+$ T cells/kg. Grade III–IV acute GVHD occurred in only 10% of patients, but a significant proportion were on prophylactic cyclosporine (48). Together, these studies demonstrate that the safe dose of G-CSF-primed DLI without concurrent immunosuppression is in the range of 1–3 $\times$ 10^4 $CD3^+$ T cells/kg; higher doses will require concurrent immunosuppression, which may limit their efficacy.

IN VITRO T CELL MANIPULATION FOR ADOPTIVE CELLULAR THERAPY

The high frequency of alloreactive T cells relative to the frequency of anti-pathogen and anti-leukemic T cells meant that the addback of unmanipulated T cells have a low therapeutic index in haploidentical HSCT and are unlikely to confer meaningful reconstitution of protective immunity without unacceptable risk of severe GVHD. Therefore, *in vitro* T cell engineering approaches to improve safety, reduce alloreactivity, and enhance protective anti-pathogen and anti-leukemic responses following allogeneic HSCT are of particular interest in this setting. Specific approaches to mitigate the risk of GVHD include enrichment for antigen-specific T cells to selectively reconstitute pathogen-specific or leukemia-specific T cells, immunomodulation of alloreactive T cells or co-infusion of suppressor cells, and safety switch gene-modification to enable the conditional deletion of T cells in the event of GVHD or other adverse events (**Figure 1**).

ANTIGEN-SPECIFIC T CELLS

Virus-Specific T Cells

One of the earliest forms of T cell therapy is the adoptive transfer of donor-derived virus-specific T cells, which can be effective in the prevention or treatment of post-transplant CMV infection (50) and EBV-associated post-transplant lymphoproliferative disease (PTLD) (**Table 2**) (55). The early success of this approach has led to the development of T cells that can target other pathogens, including adenovirus, polyoma viruses, and aspergillus. Repeated rounds of *in vitro* antigenic stimulation to expand virus-specific memory T cells from seropositive donors remains the mainstay for this approach but other technological platforms, including immunomagnetic capture of interferon-γ-producing T cells for rapid infusion has also been successfully applied (52, 56). In the past decade, there has been a strong move from donor-derived T cells toward third-party T cells. Although third-party T cells do not engraft long-term, they are effective and have the benefits of immediate availability, lower cost per treatment and, importantly, are available to patients with seronegative donors, where virus-specific T cells cannot be generated. A full discussion on virus-specific T cells is beyond the scope of this paper and has been reviewed elsewhere (57–59), but it is important to highlight here the implication of HLA-mismatching. In haploidentical HSCT, the dominant virus-specific T cell response in the donor is sometimes restricted to a non-shared HLA, in which case it will not recognize infected recipient cells. This was illustrated by a patient with recipient-derived EBV-associated PTLD after a maternal haploidentical transplant which failed to respond to donor-derived EBV-specific T cells. It was later discovered that the dominant EBV-specific response in the maternal donor was restricted to a non-shared HLA; and subsequent infusion of third-party T cells that had EBV-specific activity restricted to the patient's HLA resulted in a complete response (60).

Leukemia-Specific T Cells

Immunogenic proteins that are differentially expressed by leukemic cells and normal hematopoietic cells can be targeted by immunotherapy. In general, the expansion of leukemia-specific T cells is significantly more challenging than that for virus-specific T cells because of their lower precursor frequency and predominance in the naïve T cell compartment, but it can nonetheless be accomplished from both healthy donors and cancer patients. One of the first targets pursued clinically is Wilms tumor protein-1 (WT-1), which is overexpressed in a number of leukemias and solid tumors and encode a range of immunogenic peptide epitopes that can be used to successfully expand WT-1 specific T cells (61–63). Over the years, a number of other leukemia- and tumor-associated antigens have been identified and their epitopes mapped, including PR1 (64), BCR-ABL (65), and PRAME (66). Two early phase small-scale clinical trials have shown the feasibility of using WT-1 specific donor T cells in allogeneic stem cell transplant, and disease response was reported in two patients, one of whom had a prolonged remission (67, 68). Although mapped epitopes provide the first proof-of-principle, it is also feasible to generate antigen-specific T cells from healthy donors using overlapping peptide libraries, which does not require prior epitope-mapping or knowledge of HLA-restriction (69). However, as is the case with virus-specific T cells, HLA-restriction remains a critical consideration in haploidentical transplantation as around half of the T cell response is anticipated to be restricted to non-shared HLA.

T Cells Targeting Minor Histocompatibility Antigens

Minor histocompatibility antigens are polymorphic peptides that are presented on MHC. In the allogeneic transplant setting, minor histocompatibility antigens are most commonly the result of single nucleotide polymorphism (SNP) that differs between donor and recipients (70). Some of these antigens demonstrate preferential expression on hematopoietic cells, which make them promising targets for immunotherapy after allogeneic transplant. One such candidate is HA-1, the immunogenic form of which is presented by HLA-A2. Around half of the European population carry the immunogenic HA-1 allele (71), and if they are also HLA-A2(+), they may benefit from the adoptive transfer of

TABLE 1 | Selected studies using G-CSF primed peripheral blood mononuclear cell (G-PBMC) add-back following haploidentical HSCT.

Underlying disease	Transplant protocol	Study cohort	T cell dose/time of infusion	GVHD prophylaxis after G-PBMC	Outcome	References
AML, ALL, CML, MDS, NHL	CD34+ selected stem cell graft, with ATG and pre-transplant CSA and steroids. No post-transplant immunosuppression.	Adults ($n = 12$) but only $n = 11$ received DLI	3×10^4/kg ($n = 2$), 1×10^4/kg/month $\times$ 3 doses, or 1, 3, 10×10^4/kg/month or $1–5 \times 10^5$/kg every 2 weeks ($n = 9$) (therapeutic DLI after relapse)	None	aGVHD in 2/2 at 3×10^4/kg; aGVHD grade I not requiring systemic treatment at 1×10^4/kg/month but high relapse rate; GVHD in all patients with dose-escalated DLI or therapeutic DLI. CD4 count $\geq$ 100 /μL at 6–14 months.	Lewalle et al. (46)
AML, ALL, and CML	Unmanipulated graft, *in vivo* T cell depletion with ATG, post-transplant immunosuppression	Children ($n = 13$) Adults ($n = 5$)	$0.07–4.4 \times 10^8$/kg (median 0.58×10^8/kg); Administered after leukemia relapse; $\geq$2 doses in five patients	12 patients received CSA or low-dose MTX for 2–4 weeks	aGVHD III-IV in 6/20 patients; 15 patients achieved CR at a median of 289 days after DLI; 2 year DFS: 40%	Huang et al. (47)
High-risk leukemia/lymphoma	Unmanipulated graft, *in vivo* T cell depletion with ATG, post-transplant immunosuppression	Adults ($n = 31$)	$0.4–6.9 \times 10^7$/kg (median 1.8×10^7/kg); 45–240 (median 77) days after HSCT (prophylactic)	CSA	aGVHD II-IV in 55% and aGVHD III-IV in 10% at 100 days after DLI; severe cGVHD in 18%. TRM 26% and relapse rate 33% at 2 years after DLI	Gao et al. (48)
Malignant and non-malignant diseases	CD34+ selected stem cell graft without post-transplant immunosuppression	Children ($n = 31$) Young adults ($n = 4$)	$3–5 \times 10^4$/kg; 30–42 days after HSCT (prophylactic); Rituximab given 1 day before DLI in the last 10 patients	MTX	DLI dose of 5×10^4/kg resulted in CD4 count $>$ 100/μL by 120 days in 67% of patients and aGVHD II-IV in 11%; Fatal viral and fungal infections in 11%; 2 year OS: 69% for patients in remission at transplant.	Gilman et al. (49)

aGVHD, acute graft- vs.-host disease; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CSA, cyclosporine; DFS, disease-free survival; MDS, myelodysplastic syndrome; MTX, methotrexate; NHL, non-Hodgkin's lymphoma; TRM, treatment-related mortality; OS, overall survival.

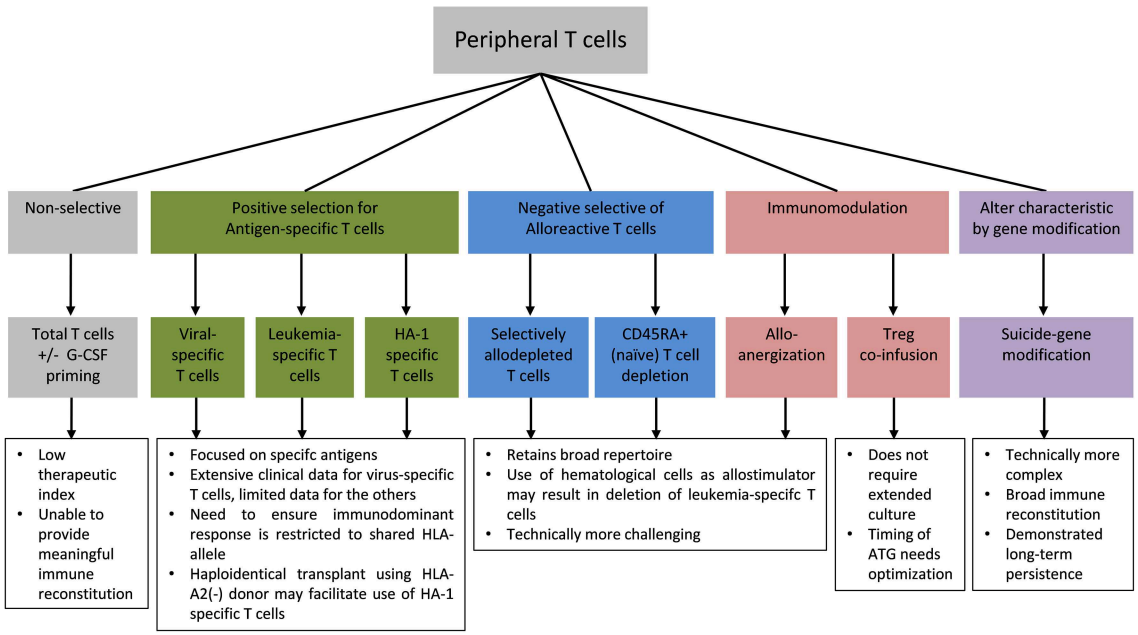

FIGURE 1 | Strategies for adoptive T cell transfer.

T cells targeting HA-1. However, it is very difficult to expand HA-1-specifc T cells from healthy donors to clinically relevant numbers because they are present in very low frequencies within the naïve T cell compartment (72). Hence, gene-modification with TCR alpha and beta chains cloned from HA-1-specific T cells has been pursued (72) and is currently undergoing clinical trial (NCT03326921). In HLA-matched setting, the donor would need to be homozygous for the non-immunogenic form of HA-1 in order to avoid fratricide of donor hematopoietic cells; but in haploidentical transplantation, the HA-1 genotype is no longer relevant if an HLA-A2(–) donor is used, thus expanding the donor pool.

SELECTIVE REMOVAL OF ALLOREACTIVE T CELLS

Selective Allodepletion and Anergy Induction

Although the adoptive transfer of antigen-specific T cells can be highly effective in specific infectious complications, they are highly targeted and do not confer broad protective immunity. A converse approach is the selective depletion or anergy induction of alloreactive T cells which, in theory, will retain broad immune repertoire minus alloreactivity (**Table 3**). The process involves the co-culture of donor T cells with irradiated recipient blood cells to activate alloreactive donor T cells: in allodepletion, activated T cells are removed based on their expression of activation markers or other properties associated with cell activation (73); and in alloanergy induction, the addition of co-stimulation blockade during co-culture results in the generation of anergic T cells (77, 81). A number of activation markers and depletion strategies have been investigated, two of which have been the focus of clinical trials. In the first method, an immunotoxin comprising a CD25-antibody conjugated to ricin is used to deplete CD25(+) alloreactive T cells *in vitro*. This could effectively reduce alloreactive T cells to <1% of the T cell population and retain responses to pathogens. The infusion of 1–8 $\times$ 10^5/kg allodepleted T cells was found to be safe in haploidentical transplant setting and could help accelerate T cell reconstitution not only numerically but also in diversity, with broad TCR repertoire and evidence of CMV and EBV-specific T cell reconstitution (73, 74). A second method involves photodynamic removal of activated T cells, which have reduced p-glycoprotein-mediated efflux of a photosensitizer, TH9402 (82). Following a phase I dose-finding study demonstrating grade I–II GVHD but no life-threatening grade III–IV GVHD (75), a multicenter phase II study was conducted. Twenty-three patients with high-risk acute leukemia were given photodepleted donor T cell products at a dose of 2 $\times$ 10^6 CD3+ cells/kg at a median of 28 days after T cell deplete haploidentical HSCT: 5 patients developed grade I-II acute GVHD and the rates of leukemia relapse and non-relapse mortality were lower compared to historical controls (76).

The induction of anergy in alloreactive donor T cells can be achieved by blocking B7/CD28 costimulation during co-culture. This can be achieved by adding CTLA-4-Ig, which is a soluble fusion protein of CTLA-4 extracellular domain to human IgG1 constant region (77), or anti-B7-1 and B7-2 antibodies during co-culture (18). This process can reduce the precursor frequency of alloreactive T cells by 1–4 logs, as measured by IL-2 production in one-way mixed lymphocyte response co-culture with irradiated recipient cells. Seminal works have demonstrated that when used with routine post-transplant

TABLE 2 | Selected studies using virus-specific T cells that enrolled predominantly haploidentical HSCT patients.

Underlying disease	Transplant protocol	Study cohort	Methodology used to generate virus-specific T cells	T cell source	T cell dose/time of infusion	Outcome	References
AML, ALL, CML, MM, lymphoma	CD34+ selected stem cell graft, myeloablative conditioning, ATG. No post-transplant immunosuppression	Adults ($n = 25$)	Repeated rounds of stimulation with CMV lysate	Stem cell donor	Escalating doses: 10^5/kg–3×10^6/kg; 13–37 days after HSCT (prophylaxis)	Reduced CMV reactivation compared to control and accelerated pathogen-specific immune reconstitution; aGVHD grade II in 1/25 patients.	Perruccio et al. (51)*
Acute leukemia and others	Haploidentical HSCT with post-transplant immunosuppression	Children ($n = 8$) Adults ($n = 3$)	IFN-γ cytokine capture for CMV-pp65 specific T cells	Stem cell donor	$2.5–16.6 \times 10^3$/kg; in patients with refractory CMV infection	Complete or partial viral clearance in 10/11 patients; No *de novo* GVHD	Feuchtinger et al. (52)†
AML/ALL and others	Unmanipulated graft, *in vivo* T cell depletion with ATG, post-transplant immunosuppression	Children and adults ($n = 32$)	Repeated rounds of stimulation with CMV-pp65 peptide mixture	Stem cell donor	$0.7–15.4 \times 10^7$/kg (median 2.7×10^7/kg); ≥2 doses in 14 patients with refractory CMV infection; 53–127 (median 69) days after HSCT	Viral clearance in 27/32 patients within 4 weeks; aGVHD grade II in 1/32 patients	Pei et al. (53)
Malignant and non-malignant diseases	Haploidentical ($n = 5$) and alternative donor ($n = 33$) HSCT	Children and adults	Multivirus-specific T cell lines	Third-party off-the-shelf	2×10^7 total cells; Treatment of infection with one ($n = 31$) or two ($n = 7$) viruses	Response rates: 94% for CMV ($n = 17$), 100% for BK virus ($n = 16$), 71% for Adenovirus ($n = 7$), 67% for HHV-6 ($n = 3$), 100% for EBV ($n = 2$). aGVHD grade I in 2/38 patients	Tzannou et al. (54)

aGVHD, acute graft- vs.-host disease; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; HHV-6, Human Herpesvirus 6; MM, multiple myeloma.

**This study also included patients who received Aspergillus-specific T cells.*

† This study also included patients who received matched unrelated or umbilical cord blood transplant.

TABLE 3 | Selected studies using allodepletion, alloanergy induction, and other immune modulation to facilitate T cell addback after haploidentical HSCT.

Underlying disease	Transplant protocol	Study cohort	Method for generating T cell therapy product	T cell dose/time of infusion	Outcome	References
Malignant and non-malignant diseases	CD34+ selected graft, myeloablative conditioning, ATG. No post-transplant immunosuppression	Infants or young children ($n = 15$)	Anti-CD25 immunotoxin-mediated allo-depletion using non-donor parent PBMC as alllo-stimulators	1–8 × 10^5/kg in dose-escalating cohorts; 15–47 days after HSCT	CD4 count ≥200/μL after 13 weeks (median) in 10 evaluable patients; massive expansion of T cells within 4 weeks of T cell infusion in three patients with CMV infection; aGVHD II-IV in 0/15 patients.	Andre-Schmutz et al. (73)*
Malignant and non-malignant diseases	CD34+ selected graft, myeloablative and non-myeloablative-conditioning, alemtuzumab. 7/16 patients received tacrolimus/CSA	Children ($n = 14$) Adults ($n = 2$)	Anti-CD25 immunotoxin-mediated allo-depletion using recipient EBV-LCL as allo-stimulators	Two dose levels: Level 1: 10^4/kg ($n = 8$), Level 2: 10^5/kg ($n = 8$); Days +30, +60 and +90 after HSCT	Dose level 2 significantly accelerated reconstitution of both CD4 and CD8 T cells, with CMV and EBV-specific responses observed in 4 of 6 evaluable patients at 2–4 months after HSCT; aGVHD II-IV in 2/16 patients	Amrolia et al. (74)
Malignant diseases	CD34+ selected graft, myeloablative conditioning, ATG. No post-transplant immunosuppression	Adults ($n = 19$)	Photodepletion with TH9402 using recipient PBMC as allo-stimulators	Phase I dose-finding study: 1 × 10^4/kg–5 × 10^6/kg; 28–40 days after HSCT	aGVHD I–II in 5/19 patients No aGVHD III–IV	Roy et al. (75)
High-risk AML and ALL	CD34+ selected graft, myeloablative conditioning, ATG.	Adults ($n = 23$)	Photodepletion with TH9402 using recipient PBMC as allo-stimulators	Phase II study: 2 × 10^6/kg; 28 days (median) after HSCT	aGVHD I–II in 22 %; 1 year TRM: 32%	Roy et al. (76) (Abstract)
Malignant and non-malignant diseases	Allo-anergized bone marrow with post-transplant CSA/MTX	Children ($n = 9$) Young adults ($n = 3$)	CTLA-4-Ig mediated alloanergy induction, using recipient PBMC as allo-stimulators	1.6–5.5 × 10^7/kg (contained in BM graft) on Day 0 of HSCT	CD4 count ≥400/μL by 6 months and CD4/CD8 ratio ≥ 1.4 by 7 months in all five surviving patients; Gut aGVHD in 3/11 patients; DFS at last follow-up: 5/12 patients	Guinan et al. (77)
High-risk acute leukemia or MDS	CD34+ selected graft, myeloablative conditioning, ATG. No post-transplant immunosuppression	Children ($n = 5$) Adults ($n = 11$)	Anti-B7.1-mediated alloanergy induction using PBMC from recipient or a second haploidentical donor as allo-stimulators	Escalating dose levels: Level 1: 10^3/kg, Level 2: 10^4/kg, Level 3: 10^5/kg; 35–42 days after HSCT	Functional virus-specific CD4 T cells detectable at a median of 9, 3, and 2.5 months and CD8 T cells at median of 9, 4, and 3 months in dose level 1/no DLI, dose level 2, and dose level 3, respectively; aGVHD II-IV in 5/16 patients; DFS at last follow-up: 4/16 patients	Davies et al. (78)
High-risk AML, ALL, lymphoma	CD34+ selected graft, myeloablative conditioning. No ATG or other serotherapy. No post-transplant immunosuppression.	Adults ($n = 26$)	Fresh Tregs (G-CSF mobilized) isolated by CD25-immuno-magnetic selection on Day (– 4), CD34+ selected stem cells and Tcon on Day 0.	Two dose levels: Level 1: Treg 2 × 10^6/kg + Tcon 0.5–1 × 10^6/kg ($n = 21$); Level 2: Treg 4 × 10^6/kg + Tcon 2 × 10^6/kg ($n = 5$)	CD4 count ≥100/μL and ≥ 200/μL at median of 42 (28–135) and 67 (40–146) days after HSCT, respectively; CD8 count ≥100/μL and ≥200/μL at median of 38 (19–95) and 48 (21–95) days, respectively; aGVHD II-IV in 2/26 patients; 12-month DFS: 46%	Di Ianni et al. (79)
High-risk AML and ALL	CD34+ selected graft, myeloablative conditioning. No serotherapy ($n = 25$), ATG or alemtuzumab ($n = 18$). No post-transplant immunosuppression.	Adults ($n = 43$)	As above	Treg 2.5 × 10^6/kg (mean) + Tcon 1.1 × 10^6/kg (mean)	CD4 count ≥100/μL and ≥200/μL at median of 40 (25–150) and 55 (45–160) days after HSCT, respectively; CD8 count ≥100/μL and ≥200/μL at median of 45 (18–100) and 60 (50–140) days, respectively; aGVHD II-IV in 15%; Relapse: 2/41 patients; 46-month DFS: 56%	Martelli et al. (17)†

(Continued)

TABLE 3 | Continued

Underlying disease	Transplant protocol	Study cohort	Method for generating T cell therapy product	T cell dose/time of infusion	Outcome	References
AML, ALL or lymphoma	CD34+ selected graft, myeloablative conditioning. ATG.	Adults (n = 12)	IL-10 mediated alloanergy induction using recipient CD3-depleted peripheral blood cells as allo-stimulators	T cells dose of 3–5 × 10^5/kg; 28–64 days after HSCT	CD4 count >150/μL and CD8 count >100/μL at a median of 30 (15–102) days in the four long-term survivors; aGVHD II-III in 5/12 patients; DFS at last follow-up: 4/12 patients	Bacchetta et al. (80)

aGVHD, acute graft- vs.-host disease; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; BM, bone marrow; CSA, cyclosporine; CTLA-4-Ig, cytotoxic T-lymphocyte-associated protein-4-immunoglobulin bound to human IgG1 constant region; DFS, disease-free survival; MDS, myelodysplastic syndrome; MM, multiple myeloma; MTX, methotrexate; OS, overall survival; PBMC, peripheral blood mononuclear cells; TRM, transplant related mortality.

**This study included haploidentical HSCT (n = 13) and matched unrelated HSCT (n = 3), with one patient receiving two transplants.*

†Some of the patients (n = 24) in this study have been reported in the Di Ianni study (79).

immunosuppression, haploidentical alloenergized bone marrow grafts could successfully engraft, rates of infection were low, and there were no GVHD-associated deaths (77). More recently, alloanergized T cells were used as DLI at 35–42 days after CD34-selected haploidentical HSCT; 16 patients were treated: low dose DLI (10^3 T cells/kg; $n = 4$) did not result in acute GVHD but also had little impact on T cell reconstitution, whereas higher doses (10^4-10^5 T cells/kg; $n = 12$) significantly accelerated T cell recovery, although five patients developed grade II–IV acute GVHD. Interestingly, *in vitro* alloanergy induction was found to expand $CD4^+CD25^+CD127^{low}$ Tregs within the graft but this did not impair the expansion of antigen-specific T cells *in vivo*, with patients on higher dose levels demonstrating reconstitution of adenovirus-, CMV-, and WT-1-specific T cells (78).

These early phase proof-of-concept studies on the ability of allodepletion and alloanergy induction in promoting engraftment and T cell immune reconstitution with clinically acceptable rates of GVHD are highly promising and call for further studies. One of the most critical considerations in this field is the source of recipient antigen-presenting cells, which can be limiting in heavily pre-treated leukopenic patients. This can be overcome by the use of EBV-transformed lymphoblastoid cell lines (EBV-LCL), which can be generated from small numbers of B cells and expand into large numbers (74), or the use of a second haploidentical family member as the source of stimulator cells (78). However, more difficult to overcome is the reliance on hematopoietic cells as stimulators, which may have the unwanted effect of depletion or anergization of leukemia-specific and hematopoietic-restricted minor histocompatibility antigen-specific T cells, thus reducing their graft- vs.-leukemia effect; and at the same time, the retention of tissue-specific alloreactive T cells that can mediate GVHD.

Naïve T Cell Depletion

T cells that mediate GVHD largely reside within the naïve T cell compartment (83), whereas virus-specific T cells largely reside within the memory T cell compartment. In the past few years, the immunomagnetic depletion of CD45RA(+) naïve T cells has emerged as an elegant and relatively simple method to deplete alloreactive T cells whilst retaining virus-specific responses (84, 85). In the haploidentical setting, 17 high-risk patients received T cell depleted grafts with the addition of CD45RA-depleted T cell fraction, which contained $<10^3$/kg $CD3^+CD45RA^+$ T cells and a median of 10^8/kg CD45RA(-) T cells (86): there was rapid reconstitution of memory T cells and remarkably, none of the patients developed acute GVHD. This promising approach is now undergoing further investigation (87) with several clinical trials in progress in the haploidentical setting (NCT02960646; NCT03849651; NCT02790515).

CO-INFUSION OF REGULATORY T CELL SUBSETS

Tregs are $CD25^+Foxp3^+$ CD4 T cells, which are the key mediators of peripheral tolerance. Their ability to prevent and attenuate GVHD is well-established in preclinical studies and

clinical correlative studies (32, 88, 89). Adoptively transferred Tregs can reduce the risk of GVHD associated with the add-back of Tcons (**Table 3**). In this approach, Tregs were isolated by CD25 immunomagnetic selection and 2×10^6 /kg Tregs were infused 4 days prior to the infusion of stem cell graft, which was given together with controlled numbers ($0.5–2 \times 10^6$/kg) of Tcons, without any post-transplant immunosuppression (17, 79). This approach was shown to accelerate CD4 and CD8 T cell immune reconstitution, with low rates (15%) of acute GVHD grade ≥2, and significant improvement in clinical outcome compared to historical controls. T cells specific to CMV, adenovirus, Aspergillus and other pathogens were detectable at much earlier timepoints compared to historical controls; and although infection remained a significant challenge, there were significant improvements in the rates of CMV reactivation and there were no target organ CMV disease (79). However, Tregs constitute only 5–10% of total CD4 T cells and it is often challenging to isolate Tregs in sufficient numbers and purity. Tregs can be expanded *in vitro* by thousands-fold without loss of purity and suppressive function (90, 91). Early phase clinical trials have shown that *in vitro* expanded Tregs are safe in cord blood transplantation, but their impact on GVHD is difficult to assess (92, 93) and this approach has not been reported in the haploidentical setting.

In vitro induced Tregs (iTregs) can be generated by the activation of conventional CD4 T cells in the presence of transforming growth factor-β (TGF-β) and rapamycin. Although iTregs have suppressive abilities *in vitro*, their suppression of GVHD in preclinical models requires the administration of rapamycin, without which they revert to pathogenic conventional T cells (92, 94). Type-1 regulatory T cells (Tr1 cells) is a subtype of Foxp3(–) CD4 T cells with regulatory function. They can regulate alloantigen-specific immune response via granzyme B-mediated killing of myeloid antigen-presenting cells and the production of immunomodulatory cytokines, chiefly interleukin-10 (IL-10) and TGF-β (95, 96). In preclinical models, the adoptive transfer of Tr1 cells is effective in suppressing GVHD (96). In a proof-of-concept clinical study, Tr1 cells were generated by co-culture of donor peripheral blood mononuclear cells with recipient CD3-depleted cells in the presence of IL-10 (**Table 3**). Twelve patients received delayed add-back of Tr1 cells after CD34-selected haploidentical HSCT: 7 died before day 100, the remaining 5 had accelerated immune reconstitution, but all had acute GVHD grade II–III (80). Thus, the results for these alternative regulatory T cell populations are mixed and naturally occurring Tregs which are biologically well-defined remain the most established form of immunomodulatory T cell population at present.

SAFETY SWITCH GENE-MODIFIED T CELLS

All T cell add-back strategies carry a risk of life-threatening GVHD. Although the risk is dose-related, it is not predictable for a given individual, and a cell dose that is safe for all is ineffective in haploidentical transplantation where there is a narrow therapeutic window. Safety switches, also known as suicide genes, refers to gene modification that enables the conditional elimination of infused cells and all their progenies in the setting of adverse events. This technology has potential application in a broad range of cellular therapeutics but their proof-of-concept was in allogeneic HSCT where the safety switch can be triggered and donor T cells deleted in the event of life-threatening GVHD (**Table 4**).

Safety Switch Technologies

The first clinically tested safety switch was herpes simplex virus thymidine kinase (HSVtk). This kinase catalyzes the monophosphorylation of ganciclovir and related nucleoside analogs, which is then converted by cellular kinases to di- and tri-phosphates, leading to arrest of DNA synthesis and subsequent cell death. T cells transduced with HSVtk retained their ability to mediate protective immunity *in vivo* and, in patients who developed GVHD, the HSVtk T cells could be eliminated by ganciclovir, with resolution of GVHD (104, 105). This strategy thus allows the administration of T cells with broad specificity and in numbers sufficient for mediating protective immunity. However, HSVtk as a safety switch has a number of drawbacks: the mechanism is dependent on cell cycle, thus killing can be delayed and is limited to proliferating cells; it precludes the use of ganciclovir and acyclovir as anti-virals; and it is a foreign protein which can elicit CD4 and CD8 immune response (106), although this is not universal and long-term persistence of HSVtk T cells has been reported (98).

The past decade has seen the development and clinical validation of inducible caspase 9 (iCasp9) as a safety switch. This technology is based on a cell membrane-permeable small molecule dimerizing drug, AP1903 (also known as Rimiducid), which binds with very high affinity and specificity to an engineered drug-binding domain. The drug-binding domain is derived from human FKBP12, with a single amino acid substitution from phenylalanine to valine (FKBP12-F36V) (107, 108). The iCasp9 transgene consists of FKBP12-F36V, joined via a short flexible linker to human caspase 9, without the caspase activation and recruitment domain (CARD), which is now superfluous (107). The administration of AP1903 induces dimerization of caspase 9, which activates the terminal effector caspase, caspase 3, with rapid induction of apoptosis. This system has a number of benefits over HSVtk: it is almost fully human-derived and hence much less likely to be immunogenic, it does not preclude the use of anti-virals and, importantly, the mechanism of cell death is cell-cycle independent, with >90% cell death within 30 min *in vitro* and *in vivo* (16, 107).

A couple of other safety switches based on cell surface expression of epitopes that enables their elimination by clinical monoclonal antibodies have also entered clinical trial, mainly in the area of chimeric antigen receptor (CAR) T cell therapy rather than in allogeneic HSCT. RQR8 is a relatively small transgene that encodes two epitopes: one from CD34, which binds to a clinical grade CD34 antibody for immunomagnetic selection; and a CD20 epitope, which functions as a safety switch in conjunction

TABLE 4 | Clinical trials using safety switch gene-modified T cells after haploidentical HSCT.

Underlying disease	Study/Transplant protocol	Study cohort	Method for generating T cell therapy product	T cell dose/time of infusion	Outcome	References
High-risk leukemia	CD34+ selected stem cell graft, myeloablative conditioning, ATG. No post-transplant immunosuppression	Adults ($n = 28$)	HSVtk modification of T cells	Intrapatient dose escalation at monthly interval if no GVHD: Dose level 1: 10^6/kg, Dose level 2: 10^7/kg, Dose level 3: 10^6/kg + IL-2, Dose level 4: 10^7/kg + IL-2; Starting 28 days after HSCT	HSVtk T cells engrafted in 22 patients: CD3+ count >100/μL at median of 75 (34–127) days after HSCT and 23 (13–42) days after HSVtk T cell infusion; aGVHD II-IV in 9/28, extensive cGVHD in 1/28, all resolved with ganciclovir administration	Ciceri et al. (97) Oliveira et al. (98)
High risk acute leukemia and MDS	Phase I, allodepleted T cell add-back after CD34-selected transplant. 9/10 patients received alemtuzumab for *in vivo* T cell depletion (NCT00710892)	Children ($n = 10$)	iCasp9 modification with prior T cell allodepletion using CD25-immunotoxin	Escalating doses: Dose level 1: 10^6/kg Dose level 2: 3×10^6/kg Dose level 3: 10^7/kg; 1st dose: 30–124 days after HSCT (4 patients received 2nd dose)	iCasp9 T cells: 54/μL and 63/μL at 1 and 2 years after HSCT, respectively; aGVHD I- II in 4/10 patients, all resolved with AP1903 administration	Di Stasi et al. (16) Zhou et al. (99)
Acute leukemia and other malignant diseases	Phase I, non-allodepleted T cell add-back after CD34-selected transplant. All 10 patients received alemtuzumab for *in vivo* T cell depletion (NCT01494103)	Children ($n = 9$) Adults ($n = 3$)	iCasp9 modification of T cells	Dose level 1: 10^4/kg ($n = 5$), Dose level 2: 5×10^5/kg ($n = 2$, one patient received 2nd dose), Dose level 3: 10^6/kg ($n = 2$), Dose level 4: 5×10^6/kg ($n = 3$); 1st dose: 31–82 days after HSCT	Viral-specific iCasp9 T cells were detected in eight patients; aGVHD I–II in 3/12, all resolved after AP1903 administration	Zhou et al. (100)
Acute leukemia and non-malignant diseases	Pilot followed by phase II study. TCR αβ+ T cell and CD19+ B cell depleted stem cell graft, myeloablative conditioning and ATG. No post-transplant immunosuppression NCT02065869 and EudraCT:2014-000584-41 Long term follow-up: NCT03733249 and EudraCT: 2016-003226-16	Children ($n = 108$)	iCasp9 modification of T cells (BPX-501)	Pilot ($n = 9$): dose escalation: 2.5×10^5/kg, 5×10^5/kg, 10^6/kg; Phase II: 1×10^6/kg (n =99); All on Day 0 of transplant	iCasp9 (BPX-501) T cells peaked at 9 months after infusion (mean 144/μL) and persisted for at least 2 years (mean 62/μL); Expansion of iCasp9 T cells correlates with CMV reactivation.	Merli et al. (101) (Abstract)
High-risk acute leukemia	CD34-selected stem cell graft with myeloablative conditioning and ATG for *in vivo* T cell depletion (ACTRN12614000290695)	Adults ($n = 3$)	iCasp9 modification of T cells	Dose level 1: 0.5×10^6/kg ($n = 2$), Dose level 2: 1×10^6/kg ($n = 1$) Additional doses allowed; 1st dose at 25–26 days after HSCT	One patient in DFS at >3.5 years; One patient died of relapse; One patient died of GVHD	Zhang et al. (102, 103)

aGVHD, acute graft- vs.-host disease; DFS, disease free survival; MDS, myelodysplastic syndrome.

with rituximab, a chimeric antibody which mediates antibody-dependent cytotoxicity widely used in the treatment of CD20-positive B cell malignancies (109). Another strategy is to express a truncated form of human epidermal growth factor receptor (EGFR), which enables the cells to be deleted by cetuximab, a chimeric antibody used to treat EGFR-expressing colorectal and head and neck cancer (110). Both RQR8 and truncated EGFR have been incorporated within other forms of gene therapy but there has not been a need to activate the safety switch and hence their clinical efficacy *in vivo* is yet to be demonstrated. These technologies have not been used as standalone safety switch in post-transplant T cell addback.

Clinical Experience of Safety Switch Modified T Cells in Haploidentical Transplantation

HSVtk has undergone clinical trial in HLA-matched sibling allogeneic HSCT (105, 111) and haploidentical HSCT (97). In a phase I–II multicentre study in haploidentical HSCT, patients received 10^6/kg−10^7/kg HSVtk T cells starting 28 days after myeloablative transplantation using CD34-selected stem cell graft and *in vivo* T cell depletion with ATG, without any post-transplant immunosuppression (97). Additional doses were allowed at monthly intervals in the absence of GVHD. Of the 50 enrolled patients, 28 were eligible to receive HSVtk T cells, none of whom had detectable T cells prior to HSVtk infusion. Twenty-two patients achieved T cell count >100 cells/μL within 13–42 days (median 23) after HSVtk T cell infusion, and reconstitution of EBV and CMV-specific T cells was observed. Ten patients developed acute GVHD and one developed chronic GVHD, all of which were controlled by the infusion of ganciclovir.

The iCasp9 safety switch was first tested in the haploidentical HSCT setting using donor T cells that were first allodepleted before iCasp9 transduction (112). Ten patients received 10^5-10^7 iCasp9 T cells/kg between day 30–124 after CD34-selected stem cell transplant. The iCasp9 T cells were found to engraft, expand, contribute to the reconstitution of both CD4 and CD8 T cells, and confer anti-viral immunity (16, 99). Four patients developed acute GVHD and received AP1903, which eliminated >90% of iCasp9 T cells within 30 min, with a further 0.5 log reduction in the subsequent 24 h and resolution of GVHD within 24–48 h (16). The safety of this approach led to a second clinical study using non-allodepleted iCasp9 T cells at 1×10^4–5×10^6 cells/kg (100). Again, immune reconstitution was accelerated and 4 patients developed GVHD, all of which were successfully managed with AP1903 administration. The largest study using iCasp9 T cells to date is a phase II multicenter study conducted in Italy on children who have undergone TCRαβ- and B cell-deplete haploidentical HSCT (NCT02065869). In a preliminary report, 108 patients received $0.25–1 \times 10^6$ iCasp9 T cells within 1 month of transplantation and it was shown that the iCasp9 T cells engrafted, peaked at 9 months after infusion and persisted for at least 2 years (101). Encouraged by these clinical successes, larger multicenter studies are underway in the USA and Europe (**Table 5**).

The addback of safety switch gene-modified T cells does not inhibit endogenous T cell reconstitution. Indeed, the infusion

TABLE 5 | Additional clinical trials on ClinicalTrials.gov involving iCasp9 T cell addback following haploidentical HSCT*.

Registration ID	Underlying disease	Study protocol	Study cohort	Study Locations	Posted Date/status	Comment
NCT03301168	Malignant and non-malignant diseases	Phase II, T cell add-back after TCR αβ, and B cell deplete stem cell transplant	Children	Multiple locations, USA	Oct-4-2017 Active/not recruiting	
NCT01744223	Malignant diseases	Phase I/II, T cell add-back after T cell deplete transplant	Adults	Multiple locations, USA	Dec-6-2012 Active/not recruiting	
NCT02477878; EudraCT:2015-005176-17	Malignant diseases	Phase I, treatment of relapse or minimal residual disease after allogeneic HSCT	Adults	Multiple locations, USA & Italy	Jun-23-2015 Active/not recruiting	This study includes matched related and haploidentical HSCT
NCT03459170	Malignant diseases	Phase I, treatment of relapse or minimal residual disease after allogeneic HSCT	Children	Italy	Mar-8-2018 Recruiting	This study includes matched related and haploidentical HSCT
NCT03639844	Non-malignant diseases	T cell add-back after TCR αβ and B cell deplete transplant	Children and young adults	Multiple locations, USA	Aug-21-2018	Expanded access protocol
NCT03699475	Malignant diseases	Phase II/III, TCR αβ and B cell deplete transplant with iCasp9 T cell addback vs. Haploidentical HSCT with PTCy	Children and adults	Nashville, TN and San Antonio, TX	Oct-8-2018/ Recruiting	
NCT02231710	Non-malignant diseases	Phase I, T cell add-back after T cell deplete transplant	Children and adults	Seattle, WA	Sep-4-2014 Active/not recruiting	Closed after enrolling one patient

Excludes studies listed on **Table 4.*

of HSVtk was associated with an increase in circulating TCR excision circles (TREC) and CD31$^+$ recent thymic emigrants, and an expansion of thymic tissue, which seemed to coincide with a peak in serum IL-7 level (113). Similarly, an increase in endogenous naïve T cell numbers after the infusion of iCasp9 T cells has also been reported (99). Together, these findings suggest that the transfer of safety switch gene-modified T cells can promote thymic output; but this phenomenon requires confirmation and further study.

Fate of Safety Switch Gene-Modified T Cells

Safety switch gene-modified T cells can be tracked long-term because the transgene is integrated within the cell genome and passed on to all daughter cells. The transduced T cells can be identified by PCR and, in many cases, also by flow cytometry for surface markers contained within the transgene. For example, HSVtk gene-modified T cells co-express ΔLNGFR and iCasp9 T cells co-express ΔCD19, which enable them to be readily distinguished on flow cytometry from T cells contained within the stem cell graft and new thymic emigrants. Using a combination of these techniques, safety switch gene-modified T cells have been shown to persist long-term: iCasp9 T cells can persist for at least 2–4 years (99, 101, 102), and long-term follow-up studies have demonstrated the presence of HSVtk T cells in all memory and effector T cell compartments for up to 14 years after infusion (98).

The clonal origins of safety-switch modified T cells can be tracked with high resolution by TCR analysis and transgene integration site analysis. The integration site refers to the position within the host cell genome in which the transgene has been inserted. Each transduction event results in the integration of the transgene into unique positions within the host cell genome; hence, analysis of transgene integration sites enables the identification of cells that are clonally related and provides information on the source, fate and proliferative capacity of HSVtk and iCaps9 T cells (**Figure 2**). In the HSVtk studies, there was a high level of TCR diversity in the first few months after adoptive transfer (16), but dominant clonotypes emerged over time (98). TCR and viral integration site analysis showed that these dominant clonotypes preferentially originated from stem cell memory (T_{scm}) and central memory (T_{cm}) in the infused cell product; and tracking of CMV- and Flu-specific HSVtk T cells showed that antigen exposure was a major driver of *in vivo* expansion and long-term persistence (98). Despite prior *in vitro* expansion and gene modification, safety switch gene-modified T cells retain massive proliferative capacity in response to antigen stimulation: we have shown that a single clone of iCasp9 T cell, bearing the same TCR and viral integration site, could expand 6-log fold in the context

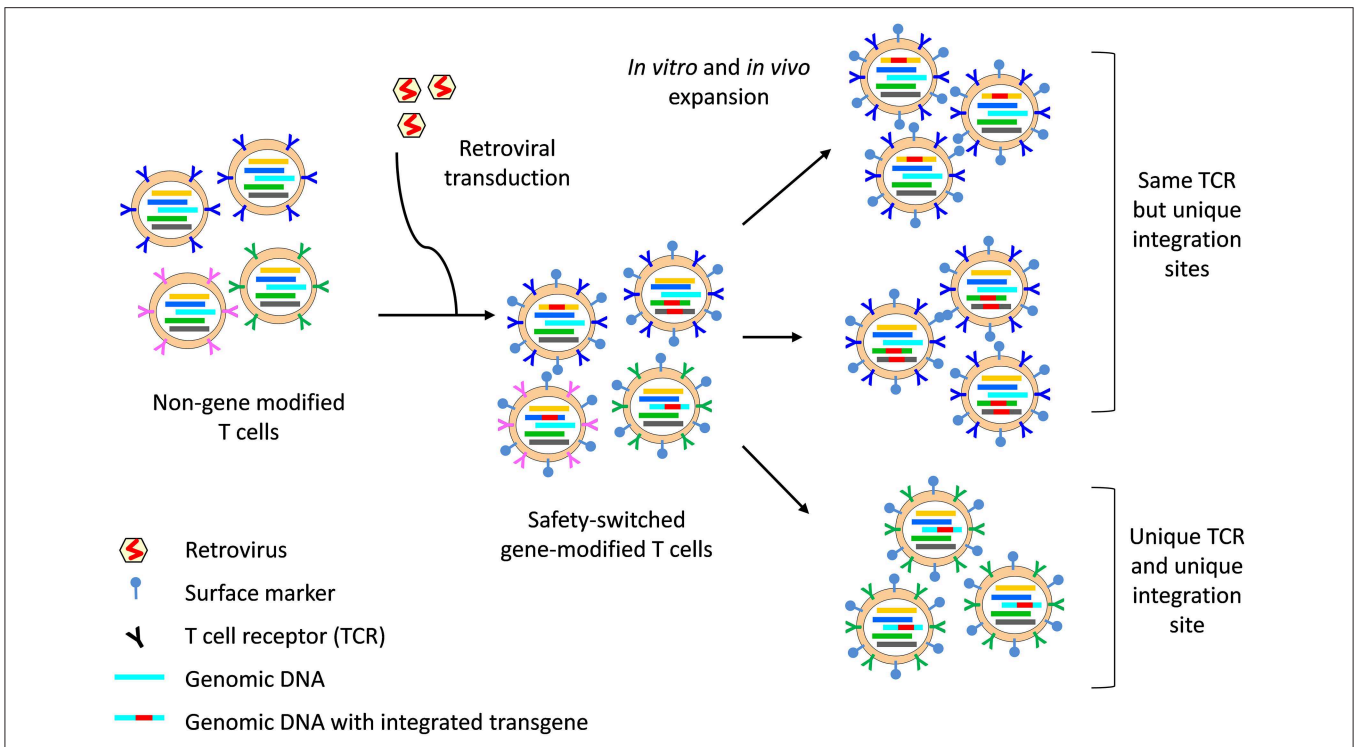

FIGURE 2 | Integration site and TCR analysis on safety switch gene-modified T cells. The safety switch transgene is integrated into the host cell genome, with 1–3 copies of integrants per cell. The region of the genomic DNA in which the transgene integrates (indicated by a unique color) is unique to each integration event and all progenies from that cell can be identified through the unique integration site. Similarly, each T cell bears a unique TCR (indicated by a unique color), which is passed on to all progenies. However, two cells bearing the same TCR may be separately gene-modified, resulting in two clones of T cells which bear the same TCR but with different integration site. Surface markers, for example ΔCD19 or ΔLNGFR, enable identification of safety switch gene-modified T cells by flow cytometry and can be used for cell sort before TCR sequencing. The concurrent analysis of TCR and integration site can provide high resolution data on the clonal origin of safety switch gene-modified T cells.

of EBV-associated PTLD and contract following resolution of EBV (102).

An interesting feature of iCasp9 safety switch system is that treatment with AP1903 preferentially deletes alloreactive T cells, and the residual iCasp9 T cells retained anti-viral specificity and could subsequently re-expand without causing GVHD (16, 99). Similarly, the kinetics of peripheral blood HSVtk was not significantly different in patients who received ganciclovir vs. those who did not, suggesting a similar phenomenon also operates in the HSVtk system (98). This preferential deletion of alloreactive T cells is in part attributed to higher level of transgene expression in activated T cells, which increase their susceptibility to safety switch activation (112, 114), whereas non-activated viral-specific T cells were relatively spared.

TIMING OF ADOPTIVE T CELL THERAPY

Adoptive T cell therapy should ideally occur as early as possible to confer protective immunity but, as explained earlier, the cytokine storm in the first 2 weeks post-transplant promotes the priming and Th1/Th17 polarization of alloreactive T cells within the cell product, thus increasingly the risk of GVHD. Furthermore, ATG or, less commonly, alemtuzumab, used during conditioning have long half-life and can eliminate the adoptively transferred T cell product if infused too early. *In vivo* T cell depletion with ATG is critical for the engraftment of haploidentical T cell deplete grafts (9, 35). It is also a standard component of the Beijing approach (11). It is not part of the PTCy approach but the addition of ATG to PTCy is undergoing clinical trial as a means to reduce the rate of GVHD (NCT03689465; NCT03608059; and NCT03367546) (115, 116). The half-life of rabbit ATG (thymoglobulin) is ∼6 days (103, 117) and most investigators wait 4–5 half-lives before T cell transfer. The half-life of alemtuzumab is around 8 days and at a standard dose of 100 mg, it will take 56 days (seven half-lives) to fall below the commonly accepted lympholytic level (118).

One approach to enable earlier T cell addback is to administer serotherapy very early, for example, the Treg study administered ATG or alemtuzumab 21 days before transplant (17). Another strategy was to use plasmapheresis prior to T cell addback: a 1–1.5 plasma volume pheresis can half the level of residual ATG (103); however, the relationship between ATG level and *in vitro* cytotoxicity is log-linear, hence halving the ATG level may have only modest biological impact and further studies to define the clinically relevant level of current ATG preparations are required.

CONCLUSIONS

Haploidentical HSCT is now widely accepted as a transplant option for patients who do not have matched sibling donors. It is particularly suitable for adoptive cellular therapy because the donor is readily available for additional donation and it is very feasible to generate advanced cellular therapeutics: sample availability, timing, and the consent process are all less of a barrier compared to using volunteer unrelated donors. In some cases, haploidentical HSCT may be preferable over HLA-matched donor if T cells targeting minor histocompatibility antigen is considered; although this is currently restricted to HA-1 (72), if successful, other hematopoietic-restricted antigens could be identified and similarly targeted (119, 120). On the other hand, it is important to consider shared vs. non-shared HLA-allele in selecting antigen-specific T cell addback after haploidentical HSCT.

The choice of T cell add-back strategy is highly dependent on local expertise and transplant platform. Proof-of-concept studies that were conducted in lymphopenic *ex vivo* T cell deplete settings may not be directly translatable to the non-lymphopenic T cell replete transplant settings. In *ex vivo* T cell deplete transplantation, T cell reconstitution is globally delayed and adoptively transferred T cells can proliferate robustly in a lymphopenic environment; hence, the most effective strategies are likely those that can reconstitute broad protective immunity, such as the infusion of allodepleted or alloanergized T cells, safety-switch gene-modified T cells, and co-infusion of Tcons with Tregs. In T cell replete transplant settings, infection is less of an issue but relapse remains a significant challenge, and strategies that are directed at relapse prevention, such as the use of leukemia-specific T cells and minor histocompatibility antigen-specific T cells may be more relevant. Rapid advances in the broader field of cellular immunotherapy will expand the armamentarium, which will likely incorporate chimeric antigen receptor T cells, off-the-shelf products and NK cell-directed therapy, all of which will help reconstitute protective immunity with an increasingly higher level of safety and efficacy after haploidentical HSCT.

AUTHOR CONTRIBUTIONS

S-KT conceived the manuscript. PZ and S-KT reviewed the literature and wrote the manuscript.

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REFERENCES

1. D'Souza A, Lee S, Zhu X, Pasquini M. Current use and trends in hematopoietic cell transplantation in the United States. *Biol Blood Marrow Transplant*. (2017) 23:1417–21. doi: 10.1016/j.bbmt.2017.05.035
2. Passweg JR, Baldomero H, Bader P, Bonini C, Duarte RF, Dufour C, et al. Use of haploidentical stem cell transplantation continues to increase: the 2015

European Society for Blood and Marrow Transplant activity survey report. *Bone Marrow Transplant*. (2017) 52:811–7. doi: 10.1038/bmt.2017.34
3. Locatelli F, Merli P, Pagliara D, Li Pira G, Falco M, Pende D, et al. Outcome of children with acute leukemia given HLA-haploidentical HSCT after alphabeta T-cell and B-cell depletion. *Blood*. (2017) 130:677–85. doi: 10.1182/blood-2017-04-779769
4. Kasamon YL, Bolanos-Meade J, Prince GT, Tsai HL, McCurdy SR, Kanakry JA, et al. Outcomes of nonmyeloablative HLA-haploidentical blood or marrow transplantation with high-dose post-transplantation cyclophosphamide in older adults. *J Clin Oncol*. (2015) 33:3152–61. doi: 10.1200/JCO.2014.60.4777
5. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, et al. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. *Blood*. (2015) 125:3956–62. doi: 10.1182/blood-2015-02-627786
6. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, et al. Haploidentical vs. matched-sibling transplant in adults with philadelphia-negative high-risk acute lymphoblastic leukemia: a biologically phase III randomized study. *Clin Cancer Res*. (2016) 22:3467–76. doi: 10.1158/1078-0432.CCR-15-2335
7. Xu LP, Jin S, Wang SQ, Xia LH, Bai H, Gao SJ, et al. Upfront haploidentical transplant for acquired severe aplastic anemia: registry-based comparison with matched related transplant. *J Hematol Oncol*. (2017) 10:25. doi: 10.1186/s13045-017-0398-y
8. Wang Y, Wang HX, Lai YR, Sun ZM, Wu DP, Jiang M, et al. Haploidentical transplant for myelodysplastic syndrome: registry-based comparison with identical sibling transplant. *Leukemia*. (2016) 30:2055–63. doi: 10.1038/leu.2016.110
9. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol*. (2005) 23:3447–54. doi: 10.1200/JCO.2005.09.117
10. O'Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, Phelps M, et al. Nonmyeloablative bone marrow transplantation from partially HLA-mismatched related donors using posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. (2002) 8:377–86. doi: 10.1053/bbmt.2002.v8.pm12171484
11. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W, et al. Haploidentical hematopoietic stem cell transplantation without *in vitro* T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant*. (2006) 38:291–7. doi: 10.1038/sj.bmt.1705445
12. Kanakry CG, Fuchs EJ, Luznik L. Modern approaches to HLA-haploidentical blood or marrow transplantation. *Nat Rev Clin Oncol*. (2016). 13:132. doi: 10.1038/nrclinonc.2015.234
13. Chang YJ, Zhao XY, Huang XJ. Immune reconstitution after haploidentical hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2014) 20:440–9. doi: 10.1016/j.bbmt.2013.11.028
14. Kanakry CG, Coffey DG, Towlerton AM, Vulic A, Storer BE, Chou J, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. *JCI Insight*. (2016) 1:e86252 doi: 10.1172/jci.insight.86252
15. DeWolf S, Sykes M. Alloimmune T cells in transplantation. *J Clin Invest*. (2017) 127:2473–81. doi: 10.1172/JCI90595
16. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med*. (2011) 365:1673–83. doi: 10.1056/NEJMoa1106152
17. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood*. (2014) 124:638–44. doi: 10.1182/blood-2014-03-564401
18. Davies JK, Gribben JG, Brennan LL, Yuk D, Nadler LM, Guinan EC. Outcome of alloanergized haploidentical bone marrow transplantation after *ex vivo* costimulatory blockade: results of 2 phase 1 studies. *Blood*. (2008) 112:2232–41. doi: 10.1182/blood-2008-03-143636
19. Bosch M, Khan FM, Storek J. Immune reconstitution after hematopoietic cell transplantation. *Curr Opin Hematol*. (2012) 19:324–35. doi: 10.1097/MOH.0b013e328353bc7d
20. Williams KM, Hakim FT, Gress RE. T cell immune reconstitution following lymphodepletion. *Semin Immunol*. (2007) 19:318–30. doi: 10.1016/j.smim.2007.10.004
21. Politikos I, Boussiotis VA. The role of the thymus in T-cell immune reconstitution after umbilical cord blood transplantation. *Blood*. (2014) 124:3201–11. doi: 10.1182/blood-2014-07-589176
22. Suessmuth Y, Mukherjee R, Watkins B, Koura DT, Finstermeier K, Desmarais C, et al. CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRbeta repertoire. *Blood*. (2015) 125:3835–50. doi: 10.1182/blood-2015-03-631853
23. Zvyagin IV, Mamedov IZ, Tatarinova OV, Komech EA, Kurnikova EE, Boyakova EV, et al. Tracking T-cell immune reconstitution after TCRalphabeta/CD19-depleted hematopoietic cells transplantation in children. *Leukemia*. (2017) 31:1145–53. doi: 10.1038/leu.2016.321
24. van Heijst JW, Ceberio I, Lipuma LB, Samilo DW, Wasilewski GD, Gonzales AM, et al. Quantitative assessment of T cell repertoire recovery after hematopoietic stem cell transplantation. *Nat Med*. (2013) 19:372–7. doi: 10.1038/nm.3100
25. Krenger W, Blazar BR, Hollander GA. Thymic T-cell development in allogeneic stem cell transplantation. *Blood*. (2011) 117:6768–76. doi: 10.1182/blood-2011-02-334623
26. Roux E, Dumont-Girard F, Starobinski M, Siegrist CA, Helg C, Chapuis B, et al. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. *Blood*. (2000) 96:2299–303.
27. Clave E, Busson M, Douay C, Peffault de Latour R, Berrou J, Rabian C, et al. Acute graft-vs.-host disease transiently impairs thymic output in young patients after allogeneic hematopoietic stem cell transplantation. *Blood*. (2009) 113:6477–84. doi: 10.1182/blood-2008-09-176594
28. Anderson G, Lane PJ, Jenkinson EJ. Generating intrathymic microenvironments to establish T-cell tolerance. *Nat Rev Immunol*. (2007) 7:954–63. doi: 10.1038/nri2187
29. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol*. (2014) 14:377–91. doi: 10.1038/nri3667
30. Teshima T, Reddy P, Liu C, Williams D, Cooke KR, Ferrara JL. Impaired thymic negative selection causes autoimmune graft-vs.-host disease. *Blood*. (2003) 102:429–35. doi: 10.1182/blood-2003-01-0266
31. Wu T, Young JS, Johnston H, Ni X, Deng R, Racine J, et al. Thymic damage, impaired negative selection, and development of chronic graft-vs.-host disease caused by donor CD4+ and CD8+ T cells. *J Immunol*. (2013) 191:488–99. doi: 10.4049/jimmunol.1300657
32. Alho AC, Kim HT, Chammas MJ, Reynolds CG, Matos TR, Forcade E, et al. Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD. *Blood*. (2016) 127:646–57. doi: 10.1182/blood-2015-10-672345
33. Morris ES, Hill GR. Advances in the understanding of acute graft-vs.-host disease. *Br J Haematol*. (2007) 137:3–19. doi: 10.1111/j.1365-2141.2007.06510.x
34. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, Anasetti C, et al. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice. *Blood*. (2011) 118:5011–20. doi: 10.1182/blood-2011-03-340315
35. Aversa F, Tabilio A, Terenzi A, Velardi A, Falzetti F, Giannoni C, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. *Blood*. (1994) 84:3948–55.
36. Colf LA, Bankovich AJ, Hanick NA, Bowerman NA, Jones LL, Kranz DM, et al. How a single T cell receptor recognizes both self and foreign MHC. *Cell*. (2007) 129:135–46. doi: 10.1016/j.cell.2007.01.048
37. Archbold JK, Macdonald WA, Miles JJ, Brennan RM, Kjer-Nielsen L, McCluskey J, et al. Alloreactivity between disparate cognate and allogeneic pMHC-I complexes is the result of highly focused, peptide-dependent structural mimicry. *J Biol Chem*. (2006) 281:34324–32. doi: 10.1074/jbc.M606755200
38. Burrows SR, Khanna R, Burrows JM, Moss DJ. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-vs.-host disease. *J Exp Med*. (1994) 179:1155–61. doi: 10.1084/jem.179.4.1155
39. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, et al. Allo-HLA reactivity of virus-specific memory T

cells is common. *Blood*. (2010) 115:3146–57. doi: 10.1182/blood-2009-07-234906
40. Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR, et al. Peripheral-blood stem cells vs. bone marrow from unrelated donors. *N Engl J Med*. (2012) 367:1487–96. doi: 10.1056/NEJMoa1203517
41. Franzke A, Piao W, Lauber J, Gatzlaff P, Konecke C, Hansen W, et al. G-CSF as immune regulator in T cells expressing the G-CSF receptor: implications for transplantation and autoimmune diseases. *Blood*. (2003) 102:734–9. doi: 10.1182/blood-2002-04-1200
42. Rutella S, Pierelli L, Bonanno G, Sica S, Ameglio F, Capoluongo E, et al. Role for granulocyte colony-stimulating factor in the generation of human T regulatory type 1 cells. *Blood*. (2002) 100:2562–71. doi: 10.1182/blood-2001-12-0291
43. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C. Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. *Blood*. (2000) 95:2484–90.
44. Hill GR, Olver SD, Kuns RD, Varelias A, Raffelt NC, Don AL, et al. Stem cell mobilization with G-CSF induces type 17 differentiation and promotes scleroderma. *Blood*. (2010) 116:819–28. doi: 10.1182/blood-2009-11-256495
45. Gartlan KH, Markey KA, Varelias A, Bunting MD, Koyama M, Kuns RD, et al. Tc17 cells are a proinflammatory, plastic lineage of pathogenic CD8+ T cells that induce GVHD without antileukemic effects. *Blood*. (2015) 126:1609–20. doi: 10.1182/blood-2015-01-622662
46. Lewalle P, Triffet A, Delforge A, Crombez P, Selleslag D, De Muynck H, et al. Donor lymphocyte infusions in adult haploidentical transplant: a dose finding study. *Bone Marrow Transpl*. (2003) 31:39–44. doi: 10.1038/sj.bmt.1703779
47. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W. Donor lymphocyte infusion for the treatment of leukemia relapse after HLA-mismatched/haploidentical T-cell-replete hematopoietic stem cell transplantation. *Haematologica*. (2007) 92:414–7. doi: 10.3324/haematol.10570
48. Gao XN, Lin J, Wang SH, Huang WR, Li F, Li HH, et al. Donor lymphocyte infusion for prevention of relapse after unmanipulated haploidentical PBSCT for very high-risk hematologic malignancies. *Ann Hematol*. (2019) 98:185–93. doi: 10.1007/s00277-018-3482-7
49. Gilman AL, Leung W, Cowan MJ, Cannon M, Epstein S, Barnhart C, et al. Donor lymphocyte infusion and methotrexate for immune recovery after T-cell depleted haploidentical transplantation. *Am J Hematol*. (2018) 93:169–78. doi: 10.1002/ajh.24949
50. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med*. (1995) 333:1038–44. doi: 10.1056/NEJM199510193331603
51. Perruccio K, Tosti A, Burchielli E, Topini F, Ruggeri L, Carotti A, et al. Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. *Blood*. (2005) 106:4397–406. doi: 10.1182/blood-2005-05-1775
52. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood*. (2010) 116:4360–7. doi: 10.1182/blood-2010-01-262089
53. Pei XY, Zhao XY, Chang YJ, Liu J, Xu LP, Wang Y, et al. Cytomegalovirus-specific T-cell transfer for refractory cytomegalovirus infection after haploidentical stem cell transplantation: the quantitative and qualitative immune recovery for cytomegalovirus. *J Infect Dis*. (2017) 216:945–56. doi: 10.1093/infdis/jix357
54. Tzannou I, Papadopoulou A, Naik S, Leung K, Martinez CA, Ramos CA, et al. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, epstein-barr virus, and adenovirus infections after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. (2017) 35:3547–57. doi: 10.1200/JCO.2017.73.0655
55. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, Krance RA, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet*. (1995) 345:9–13. doi: 10.1016/S0140-6736(95)91150-2
56. Icheva V, Kayser S, Wolff D, Tuve S, Kyzirakos C, Bethge W, et al. Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. *J Clin Oncol*. (2013) 31:39–48. doi: 10.1200/JCO.2011.39.8495
57. Bollard CM, Heslop HE. T cells for viral infections after allogeneic hematopoietic stem cell transplant. *Blood*. (2016) 127:3331–40. doi: 10.1182/blood-2016-01-628982
58. Qian C, Wang Y, Reppel L, D'Aveni M, Campidelli A, Decot V, et al. Viral-specific T-cell transfer from HSCT donor for the treatment of viral infections or diseases after HSCT. *Bone Marrow Transplant*. (2018) 53:114–22. doi: 10.1038/bmt.2017.232
59. Barrett AJ, Prockop S, Bollard CM. Virus-specific T cells: broadening applicability. *Biol Blood Marrow Transplant*. (2018) 24:13–8. doi: 10.1016/j.bbmt.2017.10.004
60. O'Reilly RJ, Hasan A, Doubrovina E, Koehne G, Prockop S. Novel strategies for adoptive therapy following HLA disparate transplants. *Best Pract Res Clin Haematol*. (2011) 24:381–91. doi: 10.1016/j.beha.2011.06.001
61. Ohminami H, Yasukawa M, Fujita S. HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. *Blood*. (2000) 95:286–93.
62. Xue SA, Gao L, Hart D, Gillmore R, Qasim W, Thrasher A, et al. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood*. (2005) 106:3062–7. doi: 10.1182/blood-2005-01-0146
63. Doubrovina ES, Doubrovin MM, Lee S, Shieh JH, Heller G, Pamer E, O'Reilly RJ. *In vitro* stimulation with WT1 peptide-loaded Epstein-Barr virus-positive B cells elicits high frequencies of WT1 peptide-specific T cells with *in vitro* and *in vivo* tumoricidal activity. *Clin Cancer Res*. (2004) 10:7207–19. doi: 10.1158/1078-0432.CCR-04-1040
64. Molldrem JJ, Lee PP, Wang C, Champlin RE, Davis MM. A PR1-human leukocyte antigen-A2 tetramer can be used to isolate low-frequency cytotoxic T lymphocytes from healthy donors that selectively lyse chronic myelogenous leukemia. *Cancer Res*. (1999) 59:2675–81.
65. Clark RE, Dodi IA, Hill SC, Lill JR, Aubert G, Macintyre AR, et al. Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. *Blood*. (2001) 98:2887–93. doi: 10.1182/blood.V98.10.2887
66. Quintarelli C, Dotti G, Hasan ST, De Angelis B, Hoyos V, Errichiello S, et al. High-avidity cytotoxic T lymphocytes specific for a new PRAME-derived peptide can target leukemic and leukemic-precursor cells. *Blood*. (2011) 117:3353–62. doi: 10.1182/blood-2010-08-300376
67. Bornhauser M, Thiede C, Platzbecker U, Kiani A, Oelschlaegel U, Babatz J, et al. Prophylactic transfer of BCR-ABL-, PR1-, and WT1-reactive donor T cells after T cell-depleted allogeneic hematopoietic cell transplantation in patients with chronic myeloid leukemia. *Blood*. (2011) 117:7174–84. doi: 10.1182/blood-2010-09-308569
68. Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM, et al. Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. *Sci Transl Med*. (2013) 5:174ra27. doi: 10.1126/scitranslmed.3004916
69. Weber G, Gerdemann U, Caruana I, Savoldo B, Hensel NF, Rabin KR, et al. Generation of multi-leukemia antigen-specific T cells to enhance the graft-vs.-leukemia effect after allogeneic stem cell transplant. *Leukemia*. (2013) 27:1538–47. doi: 10.1038/leu.2013.66
70. Martin PJ, Levine DM, Storer BE, Warren EH, Zheng X, Nelson SC, et al. Genome-wide minor histocompatibility matching as related to the risk of graft-vs.-host disease. *Blood*. (2017) 129:791–8. doi: 10.1182/blood-2016-09-737700
71. Di Terlizzi S, Zino E, Mazzi B, Magnani C, Tresoldi C, Perna SK, et al. Therapeutic and diagnostic applications of minor histocompatibility antigen HA-1 and HA-2 disparities in allogeneic hematopoietic stem cell transplantation: a survey of different populations. *Biol Blood Marrow Transplant*. (2006) 12:95–101. doi: 10.1016/j.bbmt.2005.09.017
72. Dossa RG, Cunningham T, Sommermeyer D, Medina-Rodriguez I, Biernacki MA, Foster K, et al. Development of T-cell immunotherapy for hematopoietic stem cell transplantation recipients at risk of leukemia relapse. *Blood*. (2018) 131:108–120. doi: 10.1182/blood-2017-07-791608

73. Andre-Schmutz I, Le Deist F, Hacein-Bey-Abina S, Vitetta E, Schindler J, Chedeville G, et al. Immune reconstitution without graft-vs.-host disease after haemopoietic stem-cell transplantation: a phase 1/2 study. *Lancet*. (2002) 360:130–7. doi: 10.1016/S0140-6736(02)09413-8
74. Amrolia PJ, Muccioli-Casadei G, Huls H, Adams S, Durett A, Gee A, et al. Adoptive immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical stem cell transplantation. *Blood*. (2006) 108:1797–808. doi: 10.1016/j.bbmt.2005.11.051
75. Roy DC, Lachance S, Cohen S, Delisle JS, Kiss T, Sauvageau G, et al. Allodepleted T-cell immunotherapy after haploidentical haematopoietic stem cell transplantation without severe acute graft-vs.-host disease (GVHD) in the absence of GVHD prophylaxis. *Br J Haematol*. (2019) doi: 10.1111/bjh.15970. [Epub ahead of print].
76. Roy DW, Maertens I, Lewalle J, Olavarria P, Selleslag E, Lachance D, et al. Phase II study of haploidentical stem cell transplantation using *ex vivo* photodepletion of donor lymphocyte infusions to eliminate anti-host reactivity results in low relapse rates and high survival rates: final 2 year follow-up. *Cytotherapy*. (2018) 20 (Suppl.): S10–1. doi: 10.1016/j.jcyt.2018.02.016
77. Guinan EC, Boussiotis VA, Neuberg D, Brennan LL, Hirano N, Nadler LM, et al. Transplantation of anergic histoincompatible bone marrow allografts. *N Engl J Med*. (1999) 340:1704–14. doi: 10.1056/NEJM199906033402202
78. Davies JK, Brennan LL, Wingard JR, Cogle CR, Kapoor N, Shah AJ, et al. Infusion of alloanergized donor lymphocytes after CD34-selected haploidentical myeloablative hematopoietic stem cell transplantation. *Clin Cancer Res*. (2018) 24:4098–109. doi: 10.1158/1078-0432.CCR-18-0449
79. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*. (2011) 117:3921–8. doi: 10.1182/blood-2010-10-311894
80. Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, et al. Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T cells. *Front Immunol*. (2014) 5:16. doi: 10.3389/fimmu.2014.00016
81. Gribben JG, Guinan EC, Boussiotis VA, Ke XY, Linsley L, Sieff C, et al. Complete blockade of B7 family-mediated costimulation is necessary to induce human alloantigen-specific anergy: a method to ameliorate graft-vs.-host disease and extend the donor pool. *Blood*. (1996) 87:4887–93.
82. Solomon SR, Mielke S, Savani BN, Montero A, Wisch L, Childs R, et al. Selective depletion of alloreactive donor lymphocytes: a novel method to reduce the severity of graft-vs.-host disease in older patients undergoing matched sibling donor stem cell transplantation. *Blood*. (2005) 106:1123–9. doi: 10.1182/blood-2005-01-0393
83. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, et al. Memory CD4+ T cells do not induce graft-vs.-host disease. *J Clin Invest*. (2003) 112:101–8. doi: 10.1172/JCI17601
84. Bleakley M, Heimfeld S, Jones LA, Turtle C, Krause D, Riddell SR, et al. Engineering human peripheral blood stem cell grafts that are depleted of naive T cells and retain functional pathogen-specific memory T cells. *Biol Blood Marrow Transplant*. (2014) 20:705–16. doi: 10.1016/j.bbmt.2014.01.032
85. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. *J Clin Invest*. (2015) 125:2677–89. doi: 10.1172/JCI81229
86. Triplett BM, Shook DR, Eldridge P, Li Y, Kang G, Dallas M, et al. Rapid memory T-cell reconstitution recapitulating CD45RA-depleted haploidentical transplant graft content in patients with hematologic malignancies. *Bone Marrow Transplant*. (2015) 50:1012. doi: 10.1038/bmt.2015.139
87. Maschan M, Blagov S, Shelikhova L, Shekhovtsova Z, Balashov D, Starichkova J, et al. Low-dose donor memory T-cell infusion after TCR alpha/beta depleted unrelated and haploidentical transplantation: results of a pilot trial. *Bone Marrow Transplant*. (2018) 53:264–73. doi: 10.1038/s41409-017-0035-y
88. Rieger K, Loddenkemper C, Maul J, Fietz T, Wolff D, Terpe H, et al. Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GvHD. *Blood*. (2006) 107:1717–23. doi: 10.1182/blood-2005-06-2529
89. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-vs.-host disease. *Blood*. (2005) 106:2903–11. doi: 10.1182/blood-2005-03-1257
90. Hippen KL, Merkel SC, Schirm DK, Sieben CM, Sumstad D, Kadidlo DM, et al. Massive *ex vivo* expansion of human natural regulatory T cells (T(regs)) with minimal loss of *in vivo* functional activity. *Sci Transl Med*. (2011) 3:83ra41. doi: 10.1126/scitranslmed.3001809
91. Velaga S, Alter C, Dringenberg U, Thiesler CT, Kuhs S, Olek S, et al. Clinical-grade regulatory T cells: comparative analysis of large-scale expansion conditions. *Exp Hematol*. (2017) 45:27–35 e1. doi: 10.1016/j.exphem.2016.09.008
92. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of *ex vivo* expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. (2011) 117:1061–70. doi: 10.1182/blood-2010-07-293795
93. Brunstein CG, Miller JS, McKenna DH, Hippen KL, DeFor TE, Sumstad D, et al. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood*. (2016) 127:1044–51. doi: 10.1182/blood-2015-06-653667
94. Zhang P, Tey SK, Koyama M, Kuns RD, Olver SD, Lineburg KE, et al. Induced regulatory T cells promote tolerance when stabilized by rapamycin and IL-2 *in vivo*. *J Immunol*. (2013) 191:5291–303. doi: 10.4049/jimmunol.1301181
95. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M, Gagliani N. The biology of T regulatory type 1 cells and their therapeutic application in immune-mediated diseases. *Immunity*. (2018) 49:1004–19. doi: 10.1016/j.immuni.2018.12.001
96. Zhang P, Lee JS, Gartlan KH, Schuster IS, Comerford I, Varelias A, et al. Eomesodermin promotes the development of type 1 regulatory T (TR1) cells. *Sci Immunol*. (2017) 2:eaah7152 doi: 10.1126/sciimmunol.aah7152
97. Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol*. (2009) 10:489–500. doi: 10.1016/S1470-2045(09)70074-9
98. Oliveira G, Ruggiero E, Stanghellini MT, Cieri N, D'Agostino M, Fronza R, et al. Tracking genetically engineered lymphocytes long-term reveals the dynamics of T cell immunological memory. *Sci Transl Med*. (2015) 7:317ra198. doi: 10.1126/scitranslmed.aac8265
99. Zhou X, Di Stasi A, Tey SK, Krance RA, Martinez C, Leung KS, et al. Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood*. (2014) 123:3895–905. doi: 10.1182/blood-2014-01-551671
100. Zhou X, Dotti G, Krance RA, Martinez CA, Naik S, Kamble RT, et al. Inducible caspase-9 suicide gene controls adverse effects from alloreplete T cells after haploidentical stem cell transplantation. *Blood*. (2015) 125:4103–13. doi: 10.1182/blood-2015-02-628354
101. Merli PBG, Algeri F, Sinibaldi M, Strocchio M, Li Pira L, Pagliara G, et al. Donor T cells genetically modified with a novel suicide gene (inducible caspase 9, iC9) expand and persist over time after post-allograft infusion in patients given αβ T-cell and B-cell depleted HLA-haploidentical allogeneic stem cell transplantation (αβ haplo-HSCT) contributing to accelerate immune recovery. *Blood*. (2017) 130:211.
102. Zhang P, Raju J, Ullah MA, Au R, Varelias A, Gartlan KH, et al. Phase I trial of inducible caspase 9 T cells in adult stem cell transplant demonstrates massive clonotypic proliferative potential and long-term persistence of transgenic T cells. *Clin Cancer Res*. (2019) 25:1749–55. doi: 10.1158/1078-0432.CCR-18-3069
103. Zhang P, Curley CI, Mudie K, Nakagaki M, Hill GR, Roberts JA, et al. Effect of plasmapheresis on ATG (Thymoglobulin) clearance prior to adoptive T cell transfer. *Bone Marrow Transplant*. (2019). doi: 10.1038/s41409-019-0505-5. [Epub ahead of print].
104. Traversari C, Marktel S, Magnani Z, Mangia P, Russo V, Ciceri F, et al. The potential immunogenicity of the TK suicide gene does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies. *Blood*. (2007) 109:4708–15. doi: 10.1182/blood-2006-04-015230

105. Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-vs.-leukemia. *Science*. (1997) 276:1719–24. doi: 10.1126/science.276.5319.1719
106. Berger C, Flowers ME, Warren EH, Riddell SR. Analysis of transgene-specific immune responses that limit the *in vivo* persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation. *Blood*. (2006) 107:2294–302. doi: 10.1182/blood-2005-08-3503
107. Straathof KC, Pule MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. An inducible caspase 9 safety switch for T-cell therapy. *Blood*. (2005) 105:4247–54. doi: 10.1182/blood-2004-11-4564
108. Tey SK. Adoptive T-cell therapy: adverse events and safety switches. *Clin Transl Immunol.* (2014) 3:e17. doi: 10.1038/cti.2014.11
109. Philip B, Kokalaki E, Mekkaoui L, Thomas S, Straathof K, Flutter B, et al. A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. *Blood*. (2014) 124:1277–87. doi: 10.1182/blood-2014-01-545020
110. Wang X, Chang WC, Wong CW, Colcher D, Sherman M, Ostberg JR, et al. A transgene-encoded cell surface polypeptide for selection, *in vivo* tracking, and ablation of engineered cells. *Blood*. (2011) 118:1255–63. doi: 10.1182/blood-2011-02-337360
111. Tiberghien P, Ferrand C, Lioure B, Milpied N, Angonin R, Deconinck E, et al. Administration of herpes simplex-thymidine kinase-expressing donor T cells with a T-cell-depleted allogeneic marrow graft. *Blood*. (2001) 97:63–72. doi: 10.1182/blood.V97.1.63
112. Tey SK, Dotti G, Rooney CM, Heslop HE, Brenner MK. Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation. *Biol Blood Marrow Transplant*. (2007) 13:913–24. doi: 10.1016/j.bbmt.2007.04.005
113. Vago L, Oliveira G, Bondanza A, Noviello M, Soldati C, Ghio D, et al. T-cell suicide gene therapy prompts thymic renewal in adults after hematopoietic stem cell transplantation. *Blood*. (2012) 120:1820–30. doi: 10.1182/blood-2012-01-405670
114. Chang EC, Liu H, West JA, Zhou X, Dakhova O, Wheeler DA, et al. Clonal dynamics *in vivo* of virus integration sites of T cells expressing a safety switch. *Mol Ther*. (2016) 24:736–45. doi: 10.1038/mt.2015.217
115. Chen Y, Cheng Y, Suo P, Yan C, Wang Y, Chen Y, et al. Donor-derived CD19-targeted T cell infusion induces minimal residual disease-negative remission in relapsed B-cell acute lymphoblastic leukaemia with no response to donor lymphocyte infusions after haploidentical haematopoietic stem cell transplantation. *Br J Haematol*. (2017) 179:598–605. doi: 10.1111/bjh.14923
116. Law AD, Salas MQ, Lam W, Michelis FV, Thyagu S, Kim DD, et al. Reduced-intensity conditioning and dual T lymphocyte suppression with antithymocyte globulin and post-transplant cyclophosphamide as graft-vs.-host disease prophylaxis in haploidentical hematopoietic stem cell transplants for hematological malignancies. *Biol Blood Marrow Transplant*. (2018) 24:2259–64. doi: 10.1016/j.bbmt.2018.07.008
117. Waller EK, Langston AA, Lonial S, Cherry J, Somani J, Allen AJ, et al. Pharmacokinetics and pharmacodynamics of anti-thymocyte globulin in recipients of partially HLA-matched blood hematopoietic progenitor cell transplantation. *Biol Blood Marrow Transplant*. (2003) 9:460–71. doi: 10.1016/S1083-8791(03)00127-7
118. Morris EC, Rebello P, Thomson KJ, Peggs KS, Kyriakou C, Goldstone AH, et al. Pharmacokinetics of alemtuzumab used for *in vivo* and *in vitro* T-cell depletion in allogeneic transplantations: relevance for early adoptive immunotherapy and infectious complications. *Blood*. (2003) 102:404–6. doi: 10.1182/blood-2002-09-2687
119. Marijt WA, Heemskerk MH, Kloosterboer FM, Goulmy E, Kester MG, van der Hoorn MA, et al. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci USA*. (2003) 100:2742–7. doi: 10.1073/pnas.0530192100
120. Warren EH, Fujii N, Akatsuka Y, Chaney CN, Mito JK, Loeb KR, et al. Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood*. (2010) 115:3869–78. doi: 10.1182/blood-2009-10-248997

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REVIEW
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Granulocyte Colony-Stimulating Factor-Primed Unmanipulated Haploidentical Blood and Marrow Transplantation

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Granulocyte colony-stimulating factor (G-CSF), a growth factor for neutrophils, has been successfully used for stem cell mobilization and T cell immune tolerance induction. The establishment of G-CSF-primed unmanipulated haploidentical blood and marrow transplantation (The Beijing Protocol) has achieved outcomes for the treatment of acute leukemia, myelodysplastic syndrome, and severe aplastic anemia with haploidentical allografts comparable to those of human leukocyte antigen (HLA)-matched sibling donor transplantation. Currently, G-CSF-mobilized bone marrow and/or peripheral blood stem cell sources have been widely used in unmanipulated haploidentical transplant settings. In this review, we summarize the roles of G-CSF in inducing T cell immune tolerance. We discuss the recent advances in the Beijing Protocol, mainly focusing on strategies that have been used to improve transplant outcomes in cases of poor graft function, virus infections, and relapse. The application of G-CSF-primed allografts in other haploidentical modalities is also discussed.

Keywords: haploidentical stem cell transplantation, granulocyte colony-stimulating factor, poor graft function, relapse, virus infections

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INTRODUCTION

Allogeneic stem cell transplantation (allo-SCT) is a method for the therapeutic cure of hematological malignancies (1–3). However, donor limitations restrict the wide use of allo-SCT. In the past two decades, researchers have established several haploidentical SCT (haplo-SCT) protocols based on different approaches to induce immune tolerance (4–6). Those approaches include *ex vivo* graft T cell depletion (TCD) in combination with megadoses of $CD34^+$ cells and/or anti-third-party CD8 T cells, *in vitro* CD3αβ/CD19 depletion, immune tolerance induced by granulocyte colony-stimulating factor (G-CSF) (7), and post-transplantation cyclophosphamide (PT/CY) for tolerance induction (8–18). Based on T cell tolerance induced by G-CSF, the Peking University group established a novel G-CSF-primed haploidentical blood and marrow transplantation system (The Beijing Protocol, **Figures 1**, **2**) (4, 5), including individualized conditioning regimens, the combination of unmanipulated G-CSF primed blood and marrow as allografts, donor selection based on non-human leukocyte antigen (HLA) systems, risk-directed strategies for graft-vs.-host disease (GVHD) (19), and relapse. Currently, because of the shift from TCD grafts to unmanipulated marrow and/or peripheral blood harvests, haplo-SCT is easier to

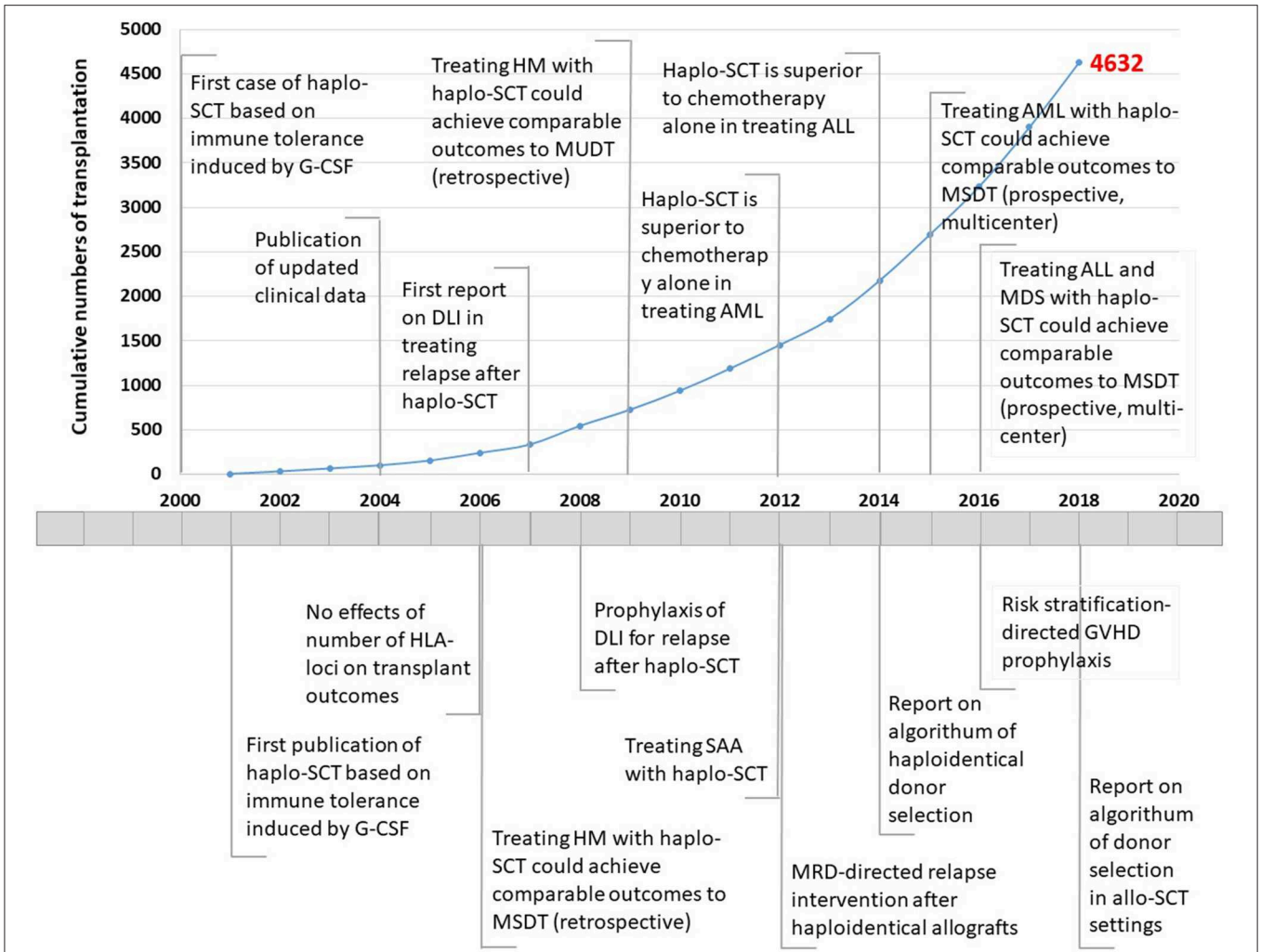

FIGURE 1 | Timeline showing the number of haploidentical stem cell transplantation and advances in Peking University Institute of Hematology, 2000–2018. Haplo-SCT, haploidentical stem cell transplantation; G-CSF, granulocyte colony-stimulating factor; HLA, human leukocyte antigen; HM, hematological malignancies; MSDT, HLA-matched sibling donor transplantation; DLI, donor lymphocyte infusion; MUDT, HLA-matched unrelated donor transplantation; SAA, severe aplastic anemia; AML, acute myeloid leukemia; MRD, minimal residual disease; ALL, acute lymphoblastic leukemia; GVHD, graft-vs.-host disease. The red number indicates cumulative cases of patients who underwent haplo-SCT until December 31, 2018.

perform than before. The development and success of haploidentical allografts worldwide makes "everyone has a donor" a reality (20). Several reviews have already been published on this topic (21–25). Here, we summarize the advances in inducing T cell tolerance by treating healthy donors with G-CSF. We discuss the recent advances in the Beijing Protocol mainly focusing on strategies that have been used for poor graft function (PGF) (26–30), virus infections (31–33), and relapse (34–36). We also indicate the application of G-CSF-primed allografts for other haploidentical allograft modalities.

T CELL TOLERANCE INDUCED BY G-CSF

G-CSF has been widely applied to mobilize hematopoietic stem/progenitor cells in allo-HSCT settings. In the past 20 years, a number of studies support the notion that G-CSF plays an important role in regulating immune cell number and function in allografts, especially in inducing T cell tolerance (37–46) Previously, researchers mainly focused on the regulatory effects of G-CSF on T cells through indirect effects, such as expanding regulatory T cells, $CD34^+$ regulatory monocytes, tolerogeneic antigen presentation cells, regulatory B cells (47), $CD3^+CD4^-CD8^-$ T cells, regulatory γδ T cells (48), suppressor interleukin-10 $(IL\text{-}10)^+$ neutrophils, myeloid-derived suppressor cells (MDSCs) (37), and granulocytic MDSCs (also known as low-density neutrophils) (49). All of these cells could suppress T cell proliferation through IL-10, transforming growth factor-β (TGF-β), nitric oxide (NO), indoleamine 2,3-dioxygenase (IDO), and/or cell contact (**Figure 3**).

In 2003, Franzke et al. (45) suggested that G-CSF acts as a strong immune regulator in T cells and directly modulates T-cell immune responses via its receptor on T cells. They demonstrated

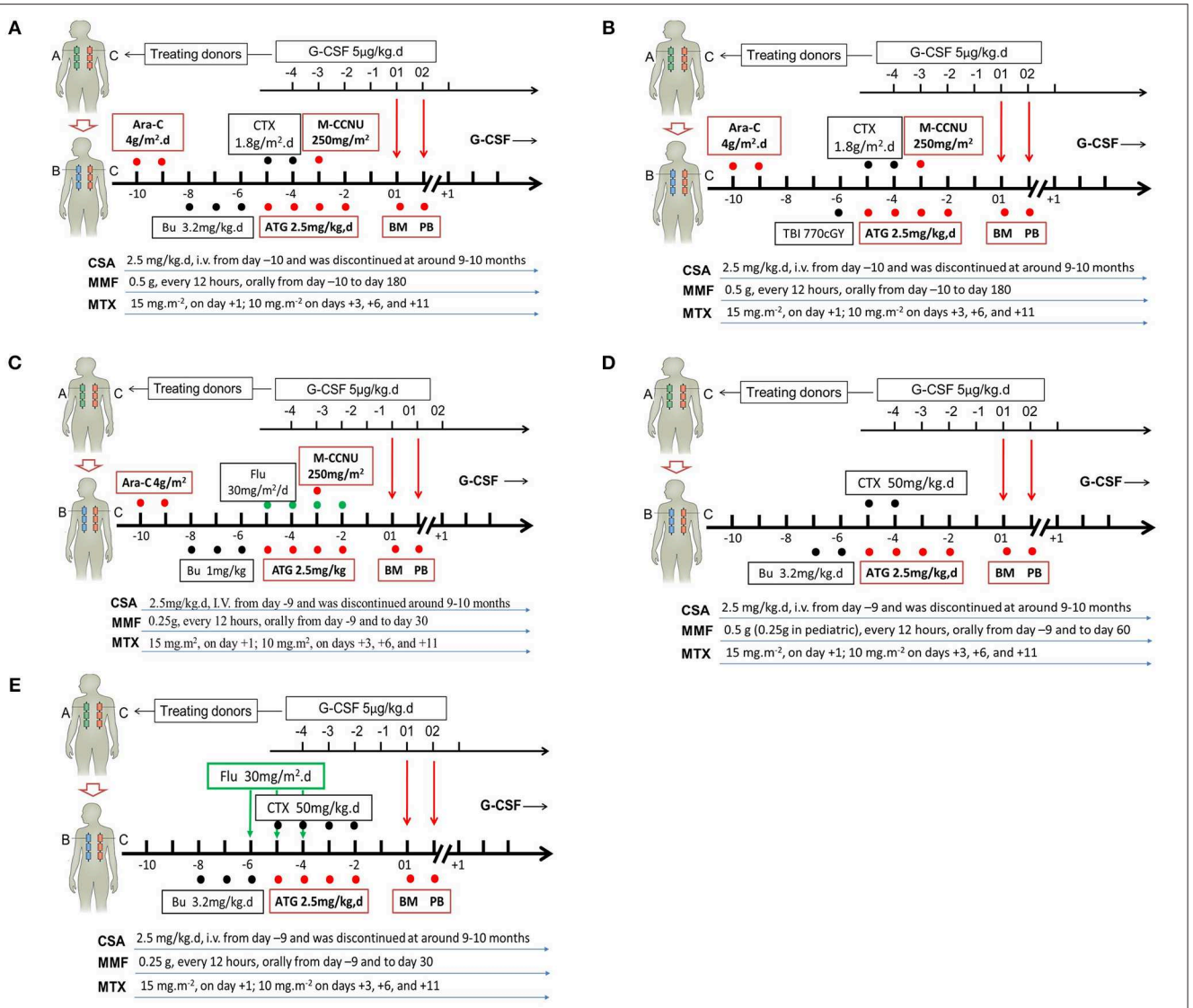

FIGURE 2 | Individual conditioning regimens in the Beijing Protocol. Conditioning regimens for hematological malignancies without total body irradiation (TBI) **(A)**, or with TBI **(B)**, or with reduced intensity **(C)**; for severe aplastic anemia **(D)**; as well as for pediatric adrenoleukodystrophy and mucopolysaccharidosis **(E)**. G-CSF, granulocyte colony-stimulating factor; Ara-C, cytrarabine; CTX, cyclophosphamide; M-CCNU, Semustine; Bu, busulfan; ATG, thymoglobulin; BM, G-CSF-primed bone marrow harvests; PB, G-CSF-mobilized peripheral blood harvests; CSA, cyclosporine; MMF, mycophenolate mofetil; MTX, methotrexate; Flu, fludarabine.

that G-CSF could limit the interferon-γ signaling in T cells by suppressing the gene expression of ISGF3-γ subunit/p48 in $CD4^+$ donor T cells. *In vitro* experiments also showed that the Th2 type could be induced by G-CSF through direct induction of GATA-3. In 2014, *in vivo* experiments also demonstrated that donor T cell alloreactivity could be modulated directly via binding to G-CSF receptor expressed on T cells (43). The authors reported that the protective effects of G-CSF on GVHD imparted during stem cell mobilization were totally dependent on direct signaling through the T cell, because WT but not G-$CSFR^{-/-}$ donor T cells were modulated by G-CSF (43). Overall, mounting evidence indicates that G-CSF can induce T cell tolerance through both indirect and direct pathways (**Figure 3**) (43, 45, 46), although the detailed molecular mechanisms of which remain unclear.

Clinically, Ringdén et al. (50) reported that application G-CSF after bone marrow transplantation increased the cumulative incidence of grades II to IV acute GVHD, which is not consistent with above-mentioned concept. The following reasons may account for this inconsistence: First, use of G-CSF after transplantation increases the levels of soluble interleukin-2 (IL-2)-receptor-alpha that may aggravate acute GVHD (51). Second, treating healthy donors with G-CSF decreases the production of tumor necrosis factor alpha, IL-2, and interferon-γ; the immunoregulatory effects of G-CSF on cytokines, T cells, and regulatory cells of donors might contribute to the lower

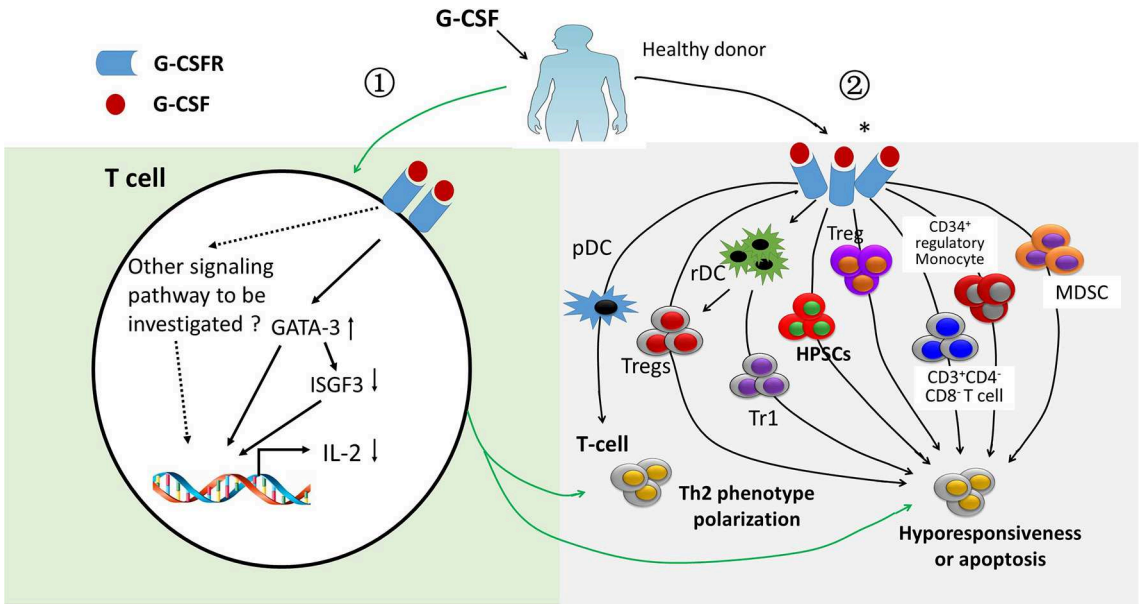

FIGURE 3 | Immune regulatory effects of treating healthy donors with granulocyte colony-stimulating factor. Granulocyte colony-stimulating factor (G-CSF) has immune regulatory effects on T cells via direct (*light green area*) and indirect mechanisms (*light gray area*). ① ISGF-3 was down-regulated and GATA3 was up-regulated by G-CSF through binding G-CSF receptor, leading to a polarization of T cells from Th1 to Th2 phenotype as well as hyporesponsiveness of T cells (*light green area*). ② Effects of G-CSF obtained from G-CSF-primed allografts on polarizing T cell from Th1 to Th2. Suppressing T cell proliferation ability by regulatory T cells (Treg), type 1 Treg, $CD34^+$ monocyte, myeloid derived suppressor cells, and $CD3^+CD4^-CD8^-$ T cells obtained from G-CSF-primed allografts either via direct contact, cytokines, such as interleukin-10 and transform growth factor-β, or via other molecules, such as arginase-1 and reactive oxygen species (*light gray area*). *indicates G-CSF receptor expressed on immune cells, such as myeloid-derived suppressor cells.

incidence of GVHD after using G-CSF-primed bone marrow harvests and/or G-CSF-mobilized peripheral blood harvests as allografts (39, 46).

RECENT ADVANCES IN THE BEIJING PROTOCOL

The Beijing Protocol, one of the main haplo-SCT modalities, has been performed in nearly 98% of transplant centers in China (52). In the first half of 2016, the proportion of haplo-SCT out of the allo-HSCT had increased to 51.7% according to data reported by Xu et al. (52) on behalf of the Chinese Blood and Marrow Transplantation Register Group. Based on the Beijing experience, Di Bartolomeo et al. (53) treated 80 patient hematological malignancies by applying a modified protocol. The key characteristics of their protocol were (i) a chemotherapy-based conditioning regimen, (ii) infusion of only G-CSF-stimulated bone marrow harvests, (iii) intensified GVHD prophylaxis with anti–thymocyte globulin (ATG), cyclosporine (CSA), methotrexate (MTX), mycophenolate mofetil (MMF), and anti-CD25 antibody. Di Bartolomeo et al. (53) found that the cumulative incidence (CI) of neutrophil engraftment was 93%. The 100-day CI for II–IV grade of acute GVHD was 24%. The 2-year CI of extensive chronic GVHD was 6%. The 3-year probability of overall survival (OS) and disease-free survival (DFS) for standard-risk and high-risk patients was 54 and 33% and 44 and 30%, respectively.

In a multicenter, prospective study, Wang et al. (54) showed that unmanipulated haplo-SCT achieved outcomes, including 3-year DFS (74 vs. 78%, $P = 0.34$) and OS (79 vs. 82%, $P = 0.36$), similar to those of HLA-matched sibling donor transplantation (MSDT) for acute myeloid leukemia patients in complete remission 1 (CR1). In another biologically phase III randomized study, Wang et al. (55) demonstrated that the 3-year DFS (61 vs. 60%, $P = 0.91$) and OS (68% vs. 66%, $P = 0.81$) were comparable between adults with Philadelphia-negative high-risk acute lymphoblastic leukemia (ALL) who underwent haplo-SCT and those who received MSDT. Similar to the report by Wang et al. (55), adults with standard-risk ALL in CR1 who underwent haplo-SCT could also achieve comparable survival to those of MSDT (56). In a registry-based study, Wang et al. (57) reported that the 4-year adjusted probabilities of OS (63 vs. 73%) and relapse-free-survival (63% vs. 71%) between patients who received haplo-SCT with 4–5/6 matched donors and those who received MSDT were comparable. These results were also confirmed by researchers from other centers in China (58). In Europe and the United States of America, a number of studies obtained similar results as reported by us (54, 55, 57), that patients with hematological malignancies who underwent haplo-SCT with PT/CY could achieve comparable outcomes to those with MSDT or HLA-matched unrelated donor transplantation (59–65).

In the past 3 years, a series of studies from different transplant centers in China confirmed the efficacy, safety, and feasibility of treating adult and pediatric severe aplastic anemia (SAA) patients

with haploidentical allografts based on G-CSF-induced immune tolerance (**Table 1**) (66–75). In a multicenter prospective study, Xu et al. (76) showed that treating SAA patients ($n = 101$) with haplo-SCT could achieve a 3-year estimated OS (89.0 vs. 91.0%, $P = 0.555$) and failure-free survival (FFS, 86.8 vs. 80.3%, $P = 0.659$) when compared with patients ($n = 48$) who received contemporaneous transplantation from matched related donors. In another registry-based study, Xu et al. (67) further observed similar 3-year estimated OS (86.1 vs. 91.3%, $P = 0.358$) and FFS (85.0 vs. 89.8%, $P = 0.413$) between the haplo-SCT and MUDT cohorts for the treatment of SAA. In this regard, Professor Neal S. Young commented that "*Haploidentical transplantation has been advocated in China as first-line treatment for children*" (77).

More recently, the G-CSF-based haplo-SCT protocol has been successfully used for the treatment of inherited metabolic storage diseases (IMD) and genetic diseases, such as adrenoleukodystrophy (ALD), mucopolysaccharidosis (MPS), and thalassaemia major (78, 79). In China, six IMD cases, including ALD ($n = 4$) and MPS ($n = 2$), were treated by Chen et al. (79) with busulfan (Bu), fludarabine (Flu), and cyclophosphamide (Cy) conditioning regimen. Hematopoietic reconstitution was achieved in all cases. Four patients developed grade I–II acute GVHD, and one patient had limited chronic GVHD. After a median follow-up of 292 days, the cumulative incidence of 1-year transplant-related mortality (TRM) was 0%. The 1-year probability of OS was 100%. In another study, Sun et al. (78) reported the results of eight thalassaemia major children who underwent haplo-SCT using the FBCA conditioning regimen [Flu, Bu, Cy, and antithymocyte globulin (ATG)]. All cases achieved hematopoietic recovery after transplantation. Four (50%) and two (25%) cases experienced grade I–II and grade III–IV acute GVHD, respectively. One patient suffered from localized chronic GVHD of the skin. All cases survived and achieved independence from blood transfusion. The OS and transfusion-free survival rates were both 100% after a median follow-up of 36 months. These studies suggest that haplo-SCT based on G-CSF could be a feasible, safe, and efficient approach for the treatment of IMD and genetic disease.

Although the indications for haplo-SCT have been extended, and no differences in the outcomes between haploidentical allografts, that is, MSDT and MUDT, were achieved, transplant complications, such as PGF (4, 26–30, 80–83) virus infection (31, 32, 84), and relapse (85–94), remain the major causes of morbidity and mortality. Fortunately, in recent years, some advances in the treatment of PGF, virus infections, and leukemia relapse after haplo-SCT have been made by scholars worldwide. We discuss these advances here.

TABLE 1 | Recent* trials and results of non-hematological malignancies treated with haploidentical allografts based on immune tolerance induced by G-CSF.

References	Pts (No.)	Diagnosis	Graft	ANC	PLT	GVHD		TRM	Relapse	FFS	OS
						Acute II–IV	cGVHD				
Xu et al. (68)	52	SAA	G-BM+G-PB	13 (10–21)	14 (7–180)	39.2%	38.1%	15.5% at 1 yr	NA	82.7 at 3 yr	84.5 at 3 yr
Xu et al. (67)	89	SAA	G-PB+G-BM (87.6%)	12 (9–20)	15 (6–91)	30.34%%	39.3% at 3 yr	NA	NA	85.0% at 3 yr	86.1% at 3 yr
Liu et al. (66)	44	SAA	G-BM+G-PB	12 (8–21)	19 (8–154)	29.3%	17.1%	NA	NA	NA	77.3% at 2 yr
Zeng et al. (75)	115	SAA	G-BM+G-PB	13 (9–25)	14 (8–82)	34.5%	18.5% at 5 yr	NA	NA	NA	74.8% at 5 yr
Sun et al. (78)	8	Thalassaemia major	G-PB	10 (10–15)	13 (10–102)	3/8	1/8	0/8	NA	100% at 1 yr	100% at 1 yr
Li et al. (65)	34	SAA	G-BM+G-PB (94.1%)	13 (10–20)	16.5 (7–30)	14.8%#	26.47%	NA	NA	93.3% at 5 yr	79.4% at 5 yr
Li et al. (69)	119	SAA	G-PB+G-BM (73%)	12 (8–22)	14 (9–154)	30%	27%	NA	NA	NA	75% at 3 yr
Lu et al. (70)	41	SAA	G-PB+G-BM	14 (10–21)	13 (3–56)	27%	39%	NA	NA	76.4% at 3 yr	80.3% at 3 yr
Chen et al. (79)	6	IMDs	G-PB+G-BM	12 (11–13)	12 (10–15)	4/6	1/6	0/6	NA	NA	100% at 1 yr
Wang et al. (72)	35	SAA	G-PB	14 (10–22)	18 (9–36)	25.71%	38.58%	14.29%	NA	NA	85.71% at 2 yr
Yang et al. (74)	20	SAA	G-PB+G-BM (50%)	16 (11–26)	19 (10–34)	40%	15%	NA	NA	80% at 3 yr	85% at 3 yr

Published between 2017 and 2019. G-CSF, granulocyte colony-stimulating factor; Pts, patients; No., number; ANC, absolute neutrophil count; PLT, platelet; GVHD, graft-vs.-host disease; cGVHD, chronic GVHD; TRM, transplant-related mortality; FFS, failure-free survival; OS, overall survival; SAA, severe aplastic anemia; G-BM, granulocyte colony-stimulating factor (G-CSF)-primed bone marrow; G-PB, G-CSF–mobilized peripheral blood stem cell grafts; NA, not available; yr, year; IMDs, inherited metabolic storage diseases.

#*indicate grades III–IV acute GVHD.*

Mechanisms and Therapies for PGF

PGF, which occurs in 5–26% of cases after allo-HSCT (26, 28–30) has become a growing obstacle that contributes to high morbidity and mortality. PGF is defined as follows: absolute neutrophil count $\leq 0.5 \times 10^9$/L, platelets $\leq 20 \times 10^9$/L, or hemoglobin ≤ 70 g/L for at least three consecutive days beyond day 28 post-transplantation with a transfusion requirement associated with hypoplastic–aplastic BM in the presence of complete donor

chimerism. Previous studies by Ciurea et al. (85) showed that patients with high donor-specific anti-HLA antibody (DSA) levels (>5,000 MFI) and complement-binding DSA antibodies (C1q positive) experienced higher risk of primary graft failure. Our data demonstrated that a number of risk factors, such as infused $CD34^+$ cells, DSA, GVHD, and CMV infection, were associated with PGF (86, 87). The available therapeutic strategies for PGF patients include the administration of hematopoietic growth factors, donor lymphocyte infusion (30), a second allo-HSCT, a $CD34^+$ cell boost (29, 30), or mesenchymal stem cell (MSC) infusion (80, 88). Additionally, eltrombopag, an oral thrombopoietin receptor agonist, has shown promising results in severe aplasia anemia. In a recent study, 12 patients who responded poorly to standard treatments for secondary PGF after allo-SHCT were treated with eltrombopag (89). The median duration from PGF diagnosis to eltrombopag treatment was 59 (range, 30–180) days. The dose of eltrombopag was 25 mg/day for 3 days and subsequently increased to 50 or 75 mg/day. After treatment for 8 (range, 2–23) weeks, 10 cases responded to eltrombopag: eight cases achieved complete response (CR) and two cases achieved partial response. The median time from treatment to achieving CR was 29 (10–49) days. The 1-year probability of OS was 83.3%. No TRM and no evidence of cataract, thrombosis, or any other grade 3/4 toxicities were observed (89). This result was also confirmed by Fu et al. (90) and Marotta et al. (91), suggesting that eltrombopag could be an alternative treatment for PGF.

Several studies have been reported seeking to elucidate the pathophysiology of PGF (4, 26, 27, 81–83). First, patients with PGF after allo-SCT had reduced and dysfunctional endothelial progenitor cells (EPCs) in the bone marrow microenvironment: these EPCs are characterized by impaired proliferation, migration, angiogenesis, and increased levels of ROS and apoptosis. In addition, the increased reactive oxygen species (ROS) could activate p38 and its downstream transcription factor in BM EPCs, both of which might contribute to the occurrence of PGF. Second, BM $CD34^+$ cells are functionally normal in PGF; however, elevated ROS in $CD34^+$ cells might lead to exhaustion of quiescent BM $CD34^+$ cells. Third, dysregulated T cell responses, including a shift in the Th1/Th2 and Tc1/Tc2 ratios toward a type 1 response and an increased Th17/Treg ratio, may also be involved in the pathogenesis of PGF. In addition, the presence of DSA may contribute to primary PGF through antibody-dependent cell-mediated cytotoxicity, resulting in impairment or apoptosis of $CD34^+$ cells in patients with PGF (92).

According to previous studies (4, 26, 27, 81–83). Shi et al. (26) and Wang et al. (27) found that atorvastatin, a regulator of p38 MAPK, may offer a novel therapeutic strategy to promote hematopoietic recovery through repair of the BM microenvironment in PGF patients. More recently, our group performed two prospective clinical trials. In the first one ($n = 68$), Kong et al. (4) found that EC < 0.1% in the BM before transplantation identified high-risk patients with PGF and PT. In the second one ($n = 35$), cases with EC < 0.1% were treated with oral *N*-acetyl-L-cysteine (NAC; 400 mg, three times per day) from −14 days to +60 days continuously (experiment group); the remaining cases with EC ≥ 0.1% ($n = 39$) underwent allo-HSCT only (control group). The authors observed a similar survival rate at 10 months after transplantation between the experiment and control groups (4). This study suggests that improvement of the BM microenvironment through EC-directed NAC intervention could be a promising approach to enhance hematopoietic recovery in allo-HSCT settings. Therefore, a randomized, controlled, multicenter study is warranted.

Prophylaxis and Treatment of Virus Infections

In haplo-SCT with G-CSF modality, the cumulative incidence of cytomegalovirus (CMV) DNAemia varies from 63.7 to 66.1%, which remains one of the main causes of morbidity and mortality. The risk factors for CMV DNAemia include HBsAg seropositivity, acute GVHD before CMV DNAemia, and poor CMV-specific $CD8^+$ T central memory subset recovery (93). In contrast, transplantation from HLA-mismatched family donors ($P < 0.001$), acute GVHD ($P < 0.001$), and donor-recipient KIR ligand mismatched ($P = 0.012$) were associated with an increased risk of refractory CMV infection (31, 32). In addition, refractory CMV infection within 60–100 days after allo-HSCT was an independent risk factor for NRM ($P = 0.015$). Compared to placebo, letermovir prophylaxis can significantly reduce the incidence of CMV disease. For cases with refractory CMV infection/reactivation or who failed ganciclovir, foscarnet, and cidofovir, these adoptive T-cell therapies, for example, CMV-specific T-cell (CMV CTL), represent a promising approach.

In a recent study, 32 patients with refractory CMV infection following haplo-SCT were treated with adoptive transfer of CMV CTL. Pei et al. (33) showed that 27 of the 32 cases exhibited CMV clearance within 4 weeks after treatment without recurrence. Compared with those of the non-refractory CMV-infected patients, the authors observed significantly fewer CMV-specific $CD8^+IFN\text{-}\gamma^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells. In addition, the CMV clearance is closely correlated with rapid and massive expansion of $CD8^+$ and $CD4^+$ CMV CTL *in vivo*. Using a humanized HCMV-infected mouse model, the same group further elucidated that systemic HCMV infection could be combated after first-line therapy with CTL through *in vivo* promotion of the recovery of graft-derived endogenous HCMV-specific CTL (84). These studies provide substantial evidence suggesting that CMV infection could be successfully addressed with prophylaxis, treatment, and adoptive transfer of CMV CTL (84). In summary, future studies should focus on the risk factor-directed intervention or development of new drugs for CMV infections in haplo-SCT settings.

Minimal Residual Disease (MRD)-Based Transplant Indication to Decrease Relapse

MRD determined by multiparameter flow cytometry (MFC) and/or real-time polymerase chain reaction (RT-PCR) at pre- and post-transplantation could be used for predicting outcomes (**Table 2**) (5, 94–105) Our group showed that, after two course consolidation therapies, patients with $t_{(8;21)}$ AML could be classified as the low-risk group or high-risk group (106).

The low-risk group was defined as cases who achieved major molecular remission (MMR)/MRD negativity [>3-log reduction in RUNX1/RUNX1T1 transcripts (<0.4%) compared with the pre-treatment baseline of 388% in our center] after the second consolidation therapy and maintained MMR for 6 months thereafter. The high-risk group was defined as cases not achieving MMR/MRD positivity after the second consolidation therapy or those exhibiting the loss of MMR (defined as RUNX1/RUNX1T1 transcript levels >0.4% in MMR patients) within 6 months of achieving MMR. In the high-risk subgroup, compared with cases receiving chemotherapy alone, cases who underwent allo-HSCT experienced a significantly lower cumulative incidence of relapse (CIR, 22.1 vs. 78.9%, $P < 0.0001$) and superior DFS (61.7 vs. 19.6%, $P = 0.001$). However, allo-HSCT was not superior to chemotherapy alone in the low-risk group. Multivariate analysis demonstrated that MRD status and treatment strategy were independent risk factors for CIR and DFS (106). Our results suggest that MRD-directed risk stratification treatment may improve the outcome not only of patients with AML with $t_{(8;21)}$ in CR1 but also of $t_{(8;21)}$ AML cases after two courses of consolidation therapy; thus, allo-HSCT should be performed for those with positive MRD.

More recently, another multicenter study enrolled 229 AML patients with NPM1-mutated (NPM1m). Balsat et al. (107) reported that a >4-log reduction in PB-MRD was significantly associated with a higher relapse incidence and shorter OS. The DFS and OS were significantly improved by allo-HSCT in those with a >4-log reduction in PB-MRD. These data suggest that NPM1m PB-MRD may be used as a predictive factor for allo-HSCT indication. Considering the comparable outcomes between haplo-SCT and MSDT, the data reported by Balsat et al. (107) and us (106) suggest that for the abovementioned MRD-positive AML patients who lack MSD, haplo-SCT might be an alternative choice to improve clinical outcomes if there are no MSDs available.

MRD-Based Donor Selection to Decrease Relapse

In the era of haplo-SCT, MSDT remains the first choice for transplant candidates according to recent literatures (23, 108). Recently, Chang et al. (109) showed that, for AML patients with positive pre-transplantation (pre-MRD), haplo-SCT could achieve lower CIR (19 vs. 57%, $P < 0.001$) and superior LFS (73 vs. 29%, $P < 0.001$) compared with those after MSDT in the retrospective group and prospective group (CIR, 13 vs. 36%, $P = 0.017$ and LFS, 80 vs. 48%, $P = 0.007$, respectively). These results were further confirmed in pediatric patients (110) and a subset of AML cases with FLT3-ITD (111), indicating superior graft-vs.-leukemia (GVL) effects of haploidentical donors to HLA-matched sibling donors. In another study, 64 Hodgkin lymphoma (HL) patients who relapsed following autologous SCT were treated with haplo-SCT with PT-Cy ($n = 30$) and MSDT ($n = 34$). After a median follow-up of 47 months, Mariotti et al. (112) found that patients receiving haplo-SCT experienced lower 3-year CIR (13 vs. 62%, $P = 0.0001$) and better PFS (60 vs. 29%, $P = 0.04$). The authors also indicated that haplo-SCT (HR, 0.17, $P = 0.02$) was independently associated with a reduced risk of relapse. In a more recent study, 151 consecutive cases with HL who were treated with haplo-SCT ($n = 61$) or MSDT ($n = 90$) were retrospectively enrolled. Gauthier et al. (113) reported a significantly lower GVHD-free/relapse-free survival (GRFS) in the MRDT group compared with those of haplo-SCT based on the PT/CY group (HR = 0.339, $P < 0.001$).

In contrast to the studies reported by other researchers and us, Ringden et al. (114) observed similar risk of relapse between acute leukemia patients who received haploidentical donor grafts ($n = 864$) and those given MSD transplants ($n = 9{,}815$), suggesting a similar GVL effect. The different results reported by Ringdén et al. and us may be related to the differences in patient population, conditioning regimen, allografts infused, and GVHD prophylaxis.

In summary, studies by others (36, 112, 113, 115) and us (109, 111) have suggested the inferiority of MSDT to haplo-SCT, indicating that, for AML patients with positive pre-MRD and HL, haploidentical donors might be selected first in experienced centers, although controversy remains. Therefore, a prospective, randomized study is needed to elucidate which one has better anti-leukemia activity, MSDT or haplo-SCT?

MRD-Directed DLI to Decrease Relapse

The transplant outcomes are worse for patients who had a hematological relapse after allo-HSCT, including haplo-SCT (116–119). In the past 10 years, a modified DLI (mDLI) protocol was used to treat patients with G-CSF-mobilized peripheral blood harvests, followed by short-term immune suppression, including cyclosporine (CSA) or methotrexate (MTX) (34, 35). Compared with traditional DLI, mDLI alleviated the pancytopenia and reduced acute GVHD without influencing the GVL effects. The safety and efficacy of mDLI for prophylaxis and treatment of relapse after haplo-SCT have been well-established. In a prospective study, 814 patients with standard-risk acute leukemia were enrolled. Yan et al. (34) reported that the MRD-positive patients who had mDLI had comparable 3-year CIR (27.8 vs. 18.1%) and DFS (55.6 vs. 61.6%) compared with those MRD-negative patients. The authors found that factors associated with lower CIR included MRD negative after transplantation (OR = 0.255, $P < 0.001$) and receiving DLI (OR = 0.269, $P < 0.001$). Factors correlated with superior DFS included receiving DLI (OR = 0.436, $P = 0.006$) and MRD negative after transplantation (OR = 0.511, $P = 0.001$). In a recent study, Yan et al. (35) further showed that, for subjects with refractory/relapsed acute leukemia, MRD- and GVHD-guided multiple DLIs could reduce CIR and improve LFS and OS.

Presently, a number of strategies, including DLI, cellular approaches (NK cells and CAR-T) (117), targeted drugs, hypomethylating agents, IFN-γ, and blinatumomab (120–122), are currently applied for relapse prevention or treatment in the clinic (116–119). Blinatumomab, a CD3 × CD19 bispecific antibody, has been approved previously for the treatment of relapsed or refractory B-cell precursor ALL (BCP-ALL). In a recent study ($n = 116$), adults with BCP-ALL in hematologic CR with MRD ($\geq 10^{-3}$) received blinatumomab 15 $\mu g/m^2$/day by continuous IV infusion for up to four cycles. Patients could

TABLE 2 | Correlation of MRD with clinical outcomes in patients who underwent Haplo-SCT.

References	Diagnosis (Pt. No.)	Transplant modalities	Methods for MRD	Transplant outcomes	Multivariate analysis
Zhao et al. (94)	ALL (543)	Haplo-SCT based on G-CSF	MFC	Positive pre-MRD, except for low level one (MRD < 0.01%), is correlated with higher CIR, and inferior LFS.	Yes
Lv et al. (5)	Intermediate risk AML (78)	Haplo-SCT based on G-CSF	MFC	Positive MRD (detectable) after two-cycle consolidation is associated with higher CIR and inferior survival.	Yes
Liu et al. (95)	AML (460)	Haplo-SCT based on G-CSF	MFC	Peri-transplantation MRD (detectable) assessment is useful for risk stratification.	Yes
Liu et al. (96)	AML (145)	Haplo-SCT based on G-CSF	MFC	Persistent positive MRD (detectable) pre-transplantation predicts poor clinical outcome.	Yes
Canaani et al. (97)	AML (393)	Haplo-SCT based on G-CSF (27.2%) Haplo-SCT with PTCy (66%) Haplo-SCT with G-CSF+PTCy (6.8%)	MFC	Positive pre-transplant MRD status (detectable) is a predictor of poor prognosis.	Yes
Qin et al. (100)	AML (14)	Haplo-SCT based on G-CSF (79%) MSDT (21%)	RT-PCR	TLS-ERG transcript levels (>1.0%) predict high-risk of relapse and inferior survival.	No
Hong et al. (104)	B-ALL (28)	Haplo-SCT based on G-CSF (90%) MSDT (10%)	TR-PCR	The E2A-PBX1 positive (detectable) after transplantation is correlated with poor prognosis.	No
Tang et al. (103)	AML (53)	Haplo-SCT based on G-CSF (75.5%) MSDT (24.5%)	RT-PCR	Post-transplant CBFB-MYH11 positive (defined as ≤3-log reduction in CBFB-MYH11 transcripts compared with the pre-treatment baseline level) could predict poor outcomes.	No
Zhao et al. (101)	T-ALL (29)	Haplo-SCT based on G-CSF (90%) MSDT (10%)	RT-PCR	Pre- or post-transplantation SIL-TAL1 positive (detectable) is associated with higher CIR and inferior DFS and OS.	No
Liu et al. (105)	AML (16)/ALL (24)	Haplo-SCT based on G-CSF (75%) MSDT (10%) Other alternative modality (15%)	RT-PCR	MLL gene positive after transplantation (detectable) is associated with higher CIR and inferior DFS and OS.	Yes
Wang et al. (102)	ALL (92)	Haplo-SCT based on G-CSF (48%) MSDT (48%) Other alternative modality (4%)	RT-PCR	Positive MRD (defined as ≤3-log reduction in RUNX1/RUNX1T1 transcripts when compared with the pre-treatment baseline level) at 1, 2, and 3 months after transplantation predicts higher CIR and inferior survival.	Yes
Zhou et al. (99)	ALL (139)	Haplo-SCT based on G-CSF (76%) MSDT (24%)	MFC	Positive MRD post-transplantation (detectable) is associated with high risk of relapse and inferior survival.	Yes
Zhou et al. (98)	AL (138)	Haplo-SCT based on G-CSF (58%) MSDT (29%) Other alternative modalities (13%)	RT-PCR	The WT1 expression level (≥0.60%) after transplantation is associated with higher CIR and inferior survival.	Yes

MRD, minimal residual disease; Haplo-SCT, haploidentical stem cell transplantation; Ref., reference; Pt., patients; No., number; G-CSF, granulocyte colony-stimulating factor; MFC, multiparameter flow cytometry; CIR, cumulative incidence of relapse; LFS, leukemia-free survival; AML, acute myeloid leukemia; PTCy, post-cyclophosphamide; RT-PCR, real-time quantitative polymerase chain reaction; MDST, human leukocyte antigen-matched sibling donor transplantation; AL, acute leukemia.

undergo allo-HSCT at any time after cycle 1. Gökbuget et al. (122) found that 88 (78%) of 113 evaluable patients achieved a complete MRD response. The RFS at 18 months was 54%. Grade 3 or 4 neurologic events occurred in 10 and 3% of cases, respectively. Four patients (3%) had cytokine release during cycle 1. These data suggest that blinatumomab could be used not only in treating relapse but only in intervention cases with positive MRD because those responders had significantly longer RFS and OS compared to non-responders. Overall, the available data (34, 35, 123) suggest that MRD-directed relapse intervention

could be a simple method in haplo-SCT settings, leading to improved outcomes.

APPLICATION OF G-CSF-PRIMED ALLOGRAFTS IN HAPLO-SCT WITH PTCy

For the unmanipulated haplo-SCT protocol with PTCy using steady-state bone marrow (SS-BM) as allografts (124), 68 patients with hematological malignancies were enrolled. The authors reported that the CIR at 1 and 2 years following transplantation was 51 and 58%, respectively. Therefore, a number of studies use G-CSF-mobilized peripheral blood harvests (G-PB) as allografts (**Table 3**) (7–17, 125). In a recent multicenter retrospective study from the United States, Bashey et al. (16) compared the outcomes of patients with hematological malignancies who underwent haplo-SCT based on PTCy receiving G-PB (n = 190) and SS-BM (n = 481). The authors found that there were no significant differences in NRM (G-PB 16% vs. SS-BM 17%, P = 0.78) and OS (G-PB 57% vs. SS-BM 54%, P = 0.52); however, compared to those with SS-BM, cases receiving G-PB experienced a significantly higher incidence of grades II–IV acute GVHD (G-PB 42% vs. SS-BM 25%, $P < 0.001$) and 2-year chronic GVHD (G-PB 41% vs. SS-BM 20%, P = 0.001). The authors demonstrated that patients receiving G-PB had a lower CIR (G-PB 28% vs. SS-BM 45%, $P < 0.001$) and superior progression-free survival (G-PB 54% vs. SS-BM 41%, P = 0.002).

More recently, a meta-analysis of four comparative retrospective reports and 10 single-arm reports, with a total of 1,759 patients (462 patients received PBSCT, and 1,297 patients received BMT) who received haplo-SCT with PTCy, was performed by Yu et al. (126) They reported that compared with those of the BM group, patients in the PB group experienced a significantly higher incidence of grade II–IV (OR = 1.778) and grade III–IV acute GVHD (OR = 1.741), as well as rapid hematopoietic recovery (OR = 1.843). No significant differences in 2-year CIR, OS, DFS, and chronic GVHD between the two groups were observed. Considering comfort, safety, and speed, G-PB is suitable for haplo-SCT and is currently widely used in the settings of haploidentical allograft with PTCy (126). Thus, multicenter, prospective, randomized studies are warranted to evaluate whether G-PB or BM is the best allograft in the setting of haplo-SCT with PTCy.

FUTURE DIRECTION

There are several questions that remain to be answered in the field of G-CSF-primed unmanipulated haploidentical blood and marrow transplantation. First, the mechanisms underlying T cell immune tolerance induced by G-CSF remain to be further investigated. Second, G-CSF-primed bone marrow harvests and/or peripheral blood stem cell harvests have been widely used in unmanipulated haploidentical allografts based on G-CSF or haplo-SCT with PTCy. However, we do not know which is the best stem cell source: G-CSF-primed bone marrow harvests, G-CSF-mobilized peripheral stem cell harvests, or a mixture of allografts of both of these harvests? Third, the

TABLE 3 | Informative trials and results regarding G-CSF-primed allografts used in haplo-SCT with PTCy.

Reference	Pts (No.)	Diagnosis	Median (range) age, yr	Conditioning regimen	Graft	GVHD		TRM	Relapse	DFS	OS
						Acute II–IV	cGVHD				
Solomon et al. (125)	20	HM	44 (25–56)	MAC	G-PB	30%	35%*	10% at 1 yr	40% at 1 yr	50% at 1 yr	69% at 1 yr
Raj et al. (9)	55	HM+SAA	49 (14–69)	RIC	G-PB	61% at 1 yr#	18% at 2 yr	23% at 2 yr	28% at 2 yr	66% at 1 yr	78% at 1 yr
Nakamae et al. (8)	20	HM	47 (18–65)	MAC	G-PB	60%	10%	11% at 1 yr	53% at 1 yr	35%* at 1 yr†	55% at 1 yr
Sugita et al. (10)	31	HM	48 (21–65)	RIC	G-PB	23%	15% at 1 yr	23% at 1 yr	45% at 1 yr	34% at 1 yr	45% at 1 yr
Jaiswal et al. (11)	20	HM	12 (2–20)	MAC	G-PB	35%	5%	20% at 1 yr	25.7% **	59.2% at 2 yr	64.3% at 2 yr
Moiseev et al. (12)	86	AML/ALL	34 (18–59)	MAC	G-PB	19%	16%	16% at 2 yr	19% at 2 yr	65% at 2 yr	69% at 2 yr
González-Llano et al. (13)	25	HM	10 (1–21)	MAC	G-PB	43%	15%	36% at 1 yr	40% at 1 yr	33% at 1 yr †	50% at 1 yr
Bashey et al. (16)	190	HM	47 (19–73)	MAC	G-PB	42% at 6 mon	41% at 2 yr	17% at 2 yr	28% at 2 yr	54% at 2 yr	57% at 2 yr
Hong et al. (14)	34	HM	11.1 (0.9–20.3)	MAC	G-PB	38.2%	9.1% at 2 yr	2.9% at 2 yr	21.7% at 2 yr	79.4% at 2 yr	85% at 2 yr
Ruggeri et al. (15)	191	AML/ALL	18.3 (1.6–50.5)	RIC (51%)	G-PB	28%	35% at 1 yr	23% at 2 yr	22% at 2 yr	51% at 2 yr	55% at 2 yr
Granata et al. (16)	181	HM	60 (19–73)	MAC	G-PB	23%	17% at 2 yr	21% at 2 yr	17% at 2 yr	62% at 2 yr	66% at 2 yr
Sugita et al. (17)	127	HM+Others	36 (17–60)	MAC (39%)	G-PB	18%	36% at 2 yr	10% at 2 yr	36% at 2 yr	54% at 2 yr	68% at 2 yr
			58 (22–65)	RIC (61%)	G-PB	14%	27% at 2 yr	20% at 2 yr	45% at 2 yr	35% at 2 yr	44% at 2 yr

Haplo-SCT, haploidentical stem cell transplantation; PTCy, post-cyclophosphamide; Pts, patients; No., number; GVHD, graft-vs.-host disease; cGVHD, chronic GVHD; TRM, transplant-related mortality; DFS, disease-free survival; OS, overall survival; HM, hematological malignancies; MAC, myeloablative conditioning regimen; G-PB, G-CSF-mobilized peripheral blood stem cell grafts; yr, year; SAA, severe aplastic anemia; RIC, reduced intensity conditioning regimen.

**indicates that the cumulative incidence of cGVHD was 35% after 20 months (range: 10–36 months) for surviving patients.*

† indicates that the estimated 1-year EFS is 35 and 33%, respectively, for these two studies.

 # indicates that the cumulative incidences of grade II and grade III acute GVHD at 1 year were 53 and 8%, respectively.

***indicates that at a median of 185 days, a cumulative incidence of disease progression was 25.7%.*

contributions of DSA, that is, impairment of the cell immune and bone marrow niche, to PGF have been identified. More efforts are needed to investigate which methods, available drugs or novel ones, could prevent or treat PGF based on known immune mechanisms underlying this complication. Fourth, more clinical data, especially multicenter, prospective, randomized trials, are needed to confirm the results that haplo-SCT has a stronger GVL effect compared with MSDT. In addition, the immunological mechanism underlying relapse after transplantation and the stronger GVL effects of haplo-SCT remain to be elucidated. Finally, regarding the MRD-directed intervention, the perfection of MRD detection methods and establishment of novel intervention strategies, such as new generation CAR-T, will further improve transplant outcomes.

In summary, several haplo-SCT protocols have been established worldwide (**Tables 1**, **3**), but each one has both disadvantages and advantages. No haplo-SCT modality could be widely accepted by each transplantation center as a standard approach for the treatment of hematological malignancies, IMD, and genetic diseases. More recently, our group demonstrated that the addition of low-dose PTCy to the Beijing Protocol can further enhance the G-CSF/ATG-induced GVHD protective activity, leading to a superior survival. A similar attempt has also been made by other researchers. In addition, with the deep understanding of the underlying mechanisms behind transplant complications, such as PGF, virus infection, and relapse, novel methods for the prevention and treatment of these complications will be established. All of these will further improve the outcomes of haplo-SCT. In this regard, well-designed prospective clinical trials are needed to compare the outcomes of the currently available haplo-SCT protocols and complication prevention and treatment methods, as well as to establish better treatments and prophylaxis for patients who had PGF, virus infection, and relapse after haploidentical allografts.

AUTHOR CONTRIBUTIONS

X-JH designed the study. X-YZ and Y-JC collected data, analyzed the data, and drafted the manuscript. All authors contributed to data interpretation, manuscript preparation, and approval of the final version.

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REFERENCES

1. Appelbaum FR. Hematopoietic-cell transplantation at 50. *N Engl J Med*. (2007) 357:1472–5. doi: 10.1056/NEJMp078166
2. Pei X, Huang X. New approaches in allogenic transplantation in AML. *Semin Hematol*. (2019) 56:147–54. doi: 10.1053/j.seminhematol.2018.08.007
3. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
4. Kong Y, Wang Y, Zhang YY, Shi MM, Mo XD, Sun YQ, et al. Prophylactic oral NAC reduced poor hematopoietic reconstitution by improving endothelial cells after haploidentical transplantation. *Blood Adv*. (2019) 3:1303–17. doi: 10.1182/bloodadvances.2018029454
5. Lv M, Wang Y, Chang YJ, Zhang XH, Xu LP, Jiang Q, et al. Myeloablative haploidentical transplantation is superior to chemotherapy for patients with intermediate-risk acute myelogenous leukemia in first complete remission. *Clin Cancer Res*. (2019) 25:1737–48. doi: 10.1158/1078-0432.CCR-18-1637
6. McCurdy SR, Kanakry JA, Showel MM, Tsai HL, Bolaños-Meade J, Rosner GL, et al. Risk-stratified outcomes of nonmyeloablative HLA-haploidentical BMT with high-dose posttransplantation cyclophosphamide. *Blood*. (2015) 125:3024–31. doi: 10.1182/blood-2015-01-623991
7. Wang Y, Wu DP, Liu QF, Xu LP, Liu KY, Zhang XH, et al. Donor and recipient age, gender and ABO incompatibility regardless of donor source: validated criteria for donor selection for haematopoietic transplants. *Leukemia*. (2018) 32:492–8. doi: 10.1038/leu.2017.199
8. Nakamae H, Koh H, Katayama T, Nishimoto M, Hayashi Y, Nakashima Y, et al. HLA haploidentical peripheral blood stem cell transplantation using reduced dose of posttransplantation cyclophosphamide for poor-prognosis or refractory leukemia and myelodysplastic syndrome. *Exp Hematol*. (2015) 43:921–9.e921. doi: 10.1016/j.exphem.2015.07.006
9. Raj K, Pagliuca A, Bradstock K, Noriega V, Potter V, Streetly M, et al. Peripheral blood hematopoietic stem cells for transplantation of hematological diseases from related, haploidentical donors after reduced-intensity conditioning. *Biol Blood Marrow Transplant*. (2014) 20:890–5. doi: 10.1016/j.bbmt.2014.03.003
10. Sugita J, Kawashima N, Fujisaki T, Kakihana K, Ota S, Matsuo K, et al. HLA-haploidentical peripheral blood stem cell transplantation with post-transplant cyclophosphamide after busulfan-containing reduced-intensity conditioning. *Biol Blood Marrow Transplant*. (2015) 21:1646–52. doi: 10.1016/j.bbmt.2015.06.008
11. Jaiswal SR, Chakrabarti A, Chatterjee S, Bhargava S, Ray K, O'Donnell P, et al. Haploidentical peripheral blood stem cell transplantation with post-transplantation cyclophosphamide in children with advanced acute leukemia with fludarabine-, busulfan-, and melphalan-based conditioning. *Biol Blood Marrow Transplant*. (2016) 22:499–504. doi: 10.1016/j.bbmt.2015.11.010
12. Moiseev IS, Pirogova OV, Alyanski AL, Babenko EV, Gindina TL, Darskaya EI, et al. Graft-versus-host disease prophylaxis in unrelated peripheral blood stem cell transplantation with post-transplantation cyclophosphamide, tacrolimus, and mycophenolate mofetil. *Biol Blood Marrow Transplant*. (2016) 22:1037–42. doi: 10.1016/j.bbmt.2016.03.004
13. Gonzalez-Llano O, Gonzalez-Lopez EE, Ramirez-Cazares AC, Marcos-Ramirez ER, Ruiz-Arguelles GJ, Gomez-Almaguer D. Haploidentical peripheral blood stem cell transplantation with posttransplant cyclophosphamide in children and adolescents with hematological malignancies. *Pediatr Blood Cancer*. (2016) 63:2033–7. doi: 10.1002/pbc.26131
14. Hong KT, Kang HJ, Choi JY, Hong CR, Cheon JE, Park JD, et al. Favorable outcome of post-transplantation cyclophosphamide haploidentical peripheral blood stem cell transplantation with targeted busulfan-based myeloablative conditioning using intensive pharmacokinetic monitoring in pediatric patients. *Biol Blood Marrow Transplant*. (2018) 24:2239–44. doi: 10.1016/j.bbmt.2018.06.034
15. Ruggeri A, Labopin M, Bacigalupo A, Gülbas Z, Koc Y, Blaise D, et al. Bone marrow versus mobilized peripheral blood stem cells in haploidentical transplants using posttransplantation cyclophosphamide. *Cancer*. (2018) 124:1428–37. doi: 10.1002/cncr.31228

16. Bashey A, Zhang MJ, McCurdy SR, St Martin A, Argall T, Anasetti C, et al. Mobilized peripheral blood stem cells versus unstimulated bone marrow as a graft source for T-cell-replete haploidentical donor transplantation using post-transplant cyclophosphamide. *J Clin Oncol*. (2017) 35:3002–9. doi: 10.1200/JCO.2017.72.8428
17. Granata A, Fürst S, Bramanti S, Legrand F, Sarina B, Harbi S, et al. Peripheral blood stem cell for haploidentical transplantation with post-transplant high dose cyclophosphamide: detailed analysis of 181 consecutive patients. *Bone Marrow Transplant*. (2019). doi: 10.1038/s41409-019-0500-x. [Epub ahead of print].
18. Sugita J, Kagaya Y, Miyamoto T, Shibasaki Y, Nagafuji K, Ota S, et al. Myeloablative and reduced-intensity conditioning in HLA-haploidentical peripheral blood stem cell transplantation using post-transplant cyclophosphamide. *Bone Marrow Transplant*. (2019) 54:432–41. doi: 10.1038/s41409-018-0279-1
19. Chang YJ, Xu LP, Wang Y, Zhang XH, Chen H, Chen YH, et al. Controlled, randomized, open-label trial of risk-stratified corticosteroid prevention of acute graft-versus-host disease after haploidentical transplantation. *J Clin Oncol*. (2016) 34:1855–63. doi: 10.1200/JCO.2015.63.8817
20. Lv M, Chang Y, Huang X. Everyone has a donor: contribution of the Chinese experience to global practice of haploidentical hematopoietic stem cell transplantation. *Front Med*. (2019) 13:45–56. doi: 10.1007/s11684-017-0595-7
21. Chang YJ, Huang XJ. Haploidentical SCT: the mechanisms underlying the crossing of HLA barriers. *Bone Marrow Transplant*. (2014) 49:873–9. doi: 10.1038/bmt.2014.19
22. Chang YJ, Huang XJ. Improving the clinical outcome of unmanipulated haploidentical blood and marrow transplantation. *Bone Marrow Transplant*. (2015) 50(Suppl 2):S21–3. doi: 10.1038/bmt.2015.90
23. Chang YJ, Luznik L, Fuchs EJ, Huang XJ. How do we choose the best donor for T-cell-replete, HLA-haploidentical transplantation? *J Hematol Oncol*. (2016) 9:35. doi: 10.1186/s13045-016-0265-2
24. Chang YJ, Huang XJ. Haploidentical hematopoietic stem cell transplantation with unmanipulated granulocyte colony stimulating factor mobilized marrow and blood grafts. *Curr Opin Hematol*. (2012) 19:454–61. doi: 10.1097/MOH.0b013e3283582322
25. Chang YJ, Huang XJ. Haploidentical stem cell transplantation: anti-thymocyte globulin-based experience. *Semin Hematol*. (2016) 53:82–9. doi: 10.1053/j.seminhematol.2016.01.004
26. Shi MM, Kong Y, Song Y, Sun YQ, Wang Y, Zhang XH, et al. Atorvastatin enhances endothelial cell function in posttransplant poor graft function. *Blood*. (2016) 128:2988–99. doi: 10.1182/blood-2016-03-702803
27. Wang YT, Kong Y, Song Y, Han W, Zhang YY, Zhang XH, et al. Increased type 1 immune response in the bone marrow immune microenvironment of patients with poor graft function after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2016) 22:1376–82. doi: 10.1016/j.bbmt.2016.04.016
28. Kong Y, Cao XN, Zhang XH, Shi MM, Lai YY, Wang Y, et al. Atorvastatin enhances bone marrow endothelial cell function in corticosteroid-resistant immune thrombocytopenia patients. *Blood*. (2018) 131:1219–33. doi: 10.1182/blood-2017-09-807248
29. Mainardi C, Ebinger M, Enkel S, Feuchtinger T, Teltschik HM, Eyrich M, et al. CD34(+) selected stem cell boosts can improve poor graft function after paediatric allogeneic stem cell transplantation. *Br J Haematol*. (2018) 180:90–9. doi: 10.1111/bjh.15012
30. Ghobadi A, Fiala MA, Ramsingh G, Gao F, Abboud CN, Stockerl-Goldstein K, et al. Fresh or cryopreserved CD34(+)-selected mobilized peripheral blood stem and progenitor cells for the treatment of poor graft function after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. (2017) 23:1072–7. doi: 10.1016/j.bbmt.2017.03.019
31. Liu J, Kong J, Chang YJ, Chen H, Chen YH, Han W, et al. Patients with refractory cytomegalovirus (CMV) infection following allogeneic haematopoietic stem cell transplantation are at high risk for CMV disease and non-relapse mortality. *Clin Microbiol Infect*. (2015) 21:1121.e9–15. doi: 10.1016/j.cmi.2015.06.009
32. Zhao XY, Luo XY, Yu XX, Zhao XS, Han TT, Chang YJ, et al. Recipient-donor KIR ligand matching prevents CMV reactivation post-haploidentical T cell-replete transplantation. *Br J Haematol*. (2017) 177:766–81. doi: 10.1111/bjh.14622
33. Pei XY, Zhao XY, Chang YJ, Liu J, Xu LP, Wang Y, et al. Cytomegalovirus-specific T-cell transfer for refractory cytomegalovirus infection after haploidentical stem cell transplantation: the quantitative and qualitative immune recovery for cytomegalovirus. *J Infect Dis*. (2017) 216:945–56. doi: 10.1093/infdis/jix357
34. Yan CH, Liu DH, Liu KY, Xu LP, Liu YR, Chen H, et al. Risk stratification-directed donor lymphocyte infusion could reduce relapse of standard-risk acute leukemia patients after allogeneic hematopoietic stem cell transplantation. *Blood*. (2012) 119:3256–62. doi: 10.1182/blood-2011-09-380386
35. Yan CH, Liu QF, Wu DP, Zhang X, Xu LP, Zhang XH, et al. Prophylactic donor lymphocyte infusion (DLI) followed by minimal residual disease and graft-versus-host disease-guided multiple DLIs could improve outcomes after allogeneic hematopoietic stem cell transplantation in patients with refractory/relapsed acute leukemia. *Biol Blood Marrow Transplant*. (2017) 23:1311–9. doi: 10.1016/j.bbmt.2017.04.028
36. Chen D, Zhou D, Guo D, Xu P, Chen B. Comparison of outcomes in hematological malignancies treated with haploidentical or HLA-identical sibling hematopoietic stem cell transplantation following myeloablative conditioning: a meta-analysis. *PLoS ONE*. (2018) 13:e0191955. doi: 10.1371/journal.pone.0191955
37. Wang K, Lv M, Chang YJ, Zhao XY, Zhao XS, Zhang YY, et al. Early myeloid-derived suppressor cells (HLA-DR(-)/(low)CD33(+)CD16(-)) expanded by granulocyte colony-stimulating factor prevent acute graft-versus-host disease (GVHD) in humanized mouse and might contribute to lower GVHD in patients post allo-HSCT. *J Hematol Oncol*. (2019) 12:31. doi: 10.1186/s13045-019-0710-0
38. Yu XX, Han TT, Xu LL, Chang YJ, Huang XJ, Zhao XY. Effect of the *in vivo* application of granulocyte colony-stimulating factor on NK cells in bone marrow and peripheral blood. *J Cell Mol Med*. (2018) 22:3025–34. doi: 10.1111/jcmm.13539
39. Perobelli SM, Mercadante AC, Galvani RG, Gonçalves-Silva T, Alves AP, Pereira-Neves A, et al. G-CSF-induced suppressor IL-10+ neutrophils promote regulatory T cells that inhibit graft-versus-host disease in a long-lasting and specific way. *J Immunol*. (2016) 197:3725–34. doi: 10.4049/jimmunol.1502023
40. D'Aveni M, Rossignol J, Coman T, Sivakumaran S, Henderson S, Manzo T, et al. G-CSF mobilizes CD34+ regulatory monocytes that inhibit graft-versus-host disease. *Sci Transl Med*. (2015) 7:281ra242. doi: 10.1126/scitranslmed.3010435
41. Morris ES, MacDonald KP, Rowe V, Johnson DH, Banovic T, Clouston AD, et al. Donor treatment with pegylated G-CSF augments the generation of IL-10-producing regulatory T cells and promotes transplantation tolerance. *Blood*. (2004) 103:3573–81. doi: 10.1182/blood-2003-08-2864
42. Melve GK, Ersvaer E, Eide GE, Kristoffersen EK, Bruserud Ø. Peripheral blood stem cell mobilization in healthy donors by granulocyte colony-stimulating factor causes preferential mobilization of lymphocyte subsets. *Front Immunol*. (2018) 9:845. doi: 10.3389/fimmu.2018.00845
43. MacDonald KP, Le Texier L, Zhang P, Morris H, Kuns RD, Lineburg KE, et al. Modification of T cell responses by stem cell mobilization requires direct signaling of the T cell by G-CSF and IL-10. *J Immunol*. (2014) 192:3180–9. doi: 10.4049/jimmunol.1302315
44. Rossetti M, Gregori S, Roncarolo MG. Granulocyte-colony stimulating factor drives the *in vitro* differentiation of human dendritic cells that induce anergy in naive T cells. *Eur J Immunol*. (2010) 40:3097–106. doi: 10.1002/eji.201040659
45. Franzke A, Piao W, Lauber J, Gatzlaff P, Könecke C, Hansen W, et al. G-CSF as immune regulator in T cells expressing the G-CSF receptor: implications for transplantation and autoimmune diseases. *Blood*. (2003) 102:734–9. doi: 10.1182/blood-2002-04-1200
46. Rutella S, Zavala F, Danese S, Kared H, Leone G. Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. *J Immunol*. (2005) 175:7085–91. doi: 10.4049/jimmunol.175.11.7085

47. Hu Y, He GL, Zhao XY, Zhao XS, Wang Y, Xu LP, et al. Regulatory B cells promote graft-versus-host disease prevention and maintain graft-versus-leukemia activity following allogeneic bone marrow transplantation. *Oncoimmunology*. (2017) 6:e1284721. doi: 10.1080/2162402X.2017.1284721
48. Xuan L, Wu X, Qiu D, Gao L, Liu H, Fan Z, et al. Regulatory gammadelta T cells induced by G-CSF participate in acute graft-versus-host disease regulation in G-CSF-mobilized allogeneic peripheral blood stem cell transplantation. *J Transl Med*. (2018) 16:144. doi: 10.1186/s12967-018-1519-2
49. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, et al. Mature CD10(+) and immature CD10(-) neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood*. (2017) 129:1343–56. doi: 10.1182/blood-2016-04-713206
50. Ringdén O, Labopin M, Gorin NC, Le Blanc K, Rocha V, Gluckman E, et al. Treatment with granulocyte colony-stimulating factor after allogeneic bone marrow transplantation for acute leukemia increases the risk of graft-versus-host disease and death: a study from the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *J Clin Oncol*. (2004) 22:416–23. doi: 10.1200/JCO.2004.06.102
51. Kobayashi S, Imamura M, Hashino S, Noto S, Mori A, Tanaka J, et al. Possible role of granulocyte colony-stimulating factor in increased serum soluble interleukin-2 receptor-alpha levels after allogeneic bone marrow transplantation. *Leuk Lymphoma*. (1999) 33:559–66. doi: 10.3109/10428199909058460
52. Xu LP, Wu DP, Han MZ, Huang H, Liu QF, Liu DH, et al. A review of hematopoietic cell transplantation in China: data and trends during 2008–2016. *Bone Marrow Transplant*. (2017) 52:1512–8. doi: 10.1038/bmt.2017.59
53. Di Bartolomeo P, Santarone S, De Angelis G, Picardi A, Cudillo L, Cerretti R, et al. Haploidentical, unmanipulated, G-CSF-primed bone marrow transplantation for patients with high-risk hematologic malignancies. *Blood*. (2013) 121:849–57. doi: 10.1182/blood-2012-08-453399
54. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, et al. Haploidentical vs. identical-sibling transplant for AML in remission: a multicenter, prospective study. *Blood*. (2015) 125:3956–62. doi: 10.1182/blood-2015-02-627786
55. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, et al. Haploidentical versus matched-sibling transplant in adults with philadelphia-negative high-risk acute lymphoblastic leukemia: a biologically phase III randomized study. *Clin Cancer Res*. (2016) 22:3467–76. doi: 10.1158/1078-0432.CCR-15-2335
56. Han LJ, Wang Y, Fan ZP, Huang F, Zhou J, Fu YW, et al. Haploidentical transplantation compared with matched sibling and unrelated donor transplantation for adults with standard-risk acute lymphoblastic leukaemia in first complete remission. *Br J Haematol*. (2017) 179:120–30. doi: 10.1111/bjh.14854
57. Wang Y, Wang HX, Lai YR, Sun ZM, Wu DP, Jiang M, et al. Haploidentical transplant for myelodysplastic syndrome: registry-based comparison with identical sibling transplant. *Leukemia*. (2016) 30:2055–63. doi: 10.1038/leu.2016.110
58. Yang B, Yu R, Cai L, Bin Guo, Chen H, Zhang H, et al. Haploidentical versus matched donor stem cell transplantation for patients with hematological malignancies: a systemic review and meta-analysis. *Bone Marrow Transplant*. (2019) 54:99–122. doi: 10.1038/s41409-018-0239-9
59. Martínez C, Gayoso J, Canals C, Finel H, Peggs K, Dominietto A, et al. Post-transplantation cyclophosphamide-based haploidentical transplantation as alternative to matched sibling or unrelated donor transplantation for hodgkin lymphoma: a registry study of the lymphoma working party of the European Society for Blood and Marrow Transplantation. *J Clin Oncol*. (2017) 35:3425–32. doi: 10.1200/JCO.2017.72.6869
60. How J, Slade M, Vu K, DiPersio JF, Westervelt P, Uy GL, et al. T cell-replete peripheral blood haploidentical hematopoietic cell transplantation with post-transplantation cyclophosphamide results in outcomes similar to transplantation from traditionally matched donors in active disease acute myeloid leukemia. *Biol Blood Marrow Transplant*. (2017) 23:648–53. doi: 10.1016/j.bbmt.2017.01.068
61. McCurdy SR, Kasamon YL, Kanakry CG, Bolaños-Meade J, Tsai HL, Showel MM, et al. Comparable composite endpoints after HLA-matched and HLA-haploidentical transplantation with post-transplantation cyclophosphamide. *Haematologica*. (2017) 102:391–400. doi: 10.3324/haematol.2016.144139
62. Ghosh N, Karmali R, Rocha V, Ahn KW, DiGilio A, Hari PN, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors versus HLA-matched sibling donors: a center for international blood and marrow transplant research analysis. *J Clin Oncol*. (2016) 34:3141–9. doi: 10.1200/JCO.2015.66.3476
63. Bashey ZA, Zhang X, Brown S, Jackson K, Morris LE, Holland HK, et al. Comparison of outcomes following transplantation with T-replete HLA-haploidentical donors using post-transplant cyclophosphamide to matched related and unrelated donors for patients with AML and MDS aged 60 years or older. *Bone Marrow Transplant*. (2018) 53:756–63. doi: 10.1038/s41409-018-0126-4
64. Brissot E, Labopin M, Ehninger G, Stelljes M, Brecht A, Ganser A, et al. Haploidentical versus unrelated allogeneic stem cell transplantation for relapsed/refractory acute myeloid leukemia: a report on 1578 patients from the Acute Leukemia Working Party of the EBMT. *Haematologica*. (2019) 104:524–32. doi: 10.3324/haematol.2017.187450
65. Li S, Wang B, Fu L, Pang Y, Zhu G, Zhou X, et al. Hematopoietic stem cell transplantation without *in vivo* T-cell depletion for pediatric aplastic anemia: a single-center experience. *Pediatr Transplant*. (2018) 22:e13204. doi: 10.1111/petr.13204
66. Liu Z, Zhang Y, Xiao H, Yao Z, Zhang H, Liu Q, et al. Cotransplantation of bone marrow-derived mesenchymal stem cells in haploidentical hematopoietic stem cell transplantation in patients with severe aplastic anemia: an interim summary for a multicenter phase II trial results. *Bone Marrow Transplant*. (2017) 52:704–10. doi: 10.1038/bmt.2016.347
67. Xu LP, Jin S, Wang SQ, Xia LH, Bai H, Gao SJ, et al. Upfront haploidentical transplant for acquired severe aplastic anemia: registry-based comparison with matched related transplant. *J Hematol Oncol*. (2017) 10:25. doi: 10.1186/s13045-017-0398-y
68. Xu LP, Zhang XH, Wang FR, Mo XD, Han TT, Han W, et al. Haploidentical transplantation for pediatric patients with acquired severe aplastic anemia. *Bone Marrow Transplant*. (2017) 52:381–7. doi: 10.1038/bmt.2016.281
69. Li Y, Duan F, Xiao H, Wu X, Wang S, Xu D, et al. Therapeutic outcomes of haploidentical allogeneic hematopoietic stem cell transplantation in patients with severe aplastic anemia: a multicenter study. *Transplantation*. (2018) 102:1724–31. doi: 10.1097/TP.0000000000002200
70. Lu Y, Sun RJ, Zhao YL, Xiong M, Cao XY, Zhang JP, et al. Unmanipulated haploidentical hematopoietic stem cell transplantation achieved outcomes comparable with matched unrelated donor transplantation in young acquired severe aplastic anemia. *Biol Blood Marrow Transplant*. (2018) 24:1881–7. doi: 10.1016/j.bbmt.2018.05.015
71. Yue C, Ding Y, Gao Y, Li L, Pang Y, Liu Z, et al. Cotransplantation of haploidentical hematopoietic stem cells and allogeneic bone marrow-derived mesenchymal stromal cells as a first-line treatment in very severe aplastic anemia patients with refractory infections. *Eur J Haematol*. (2018) 100:624–9. doi: 10.1111/ejh.13060
72. Wang Z, Yu H, Cao F, Liu Z, Liu Z, Feng W, et al. Donor-derived marrow mesenchymal stromal cell co-transplantation following a haploidentical hematopoietic stem cell transplantation trail to treat severe aplastic anemia in children. *Ann Hematol*. (2019) 98:473–9. doi: 10.1007/s00277-018-3523-2
73. Xu ZL, Zhou M, Jia JS, Mo WJ, Zhang XH, Zhang YP, et al. Immunosuppressive therapy versus haploidentical transplantation in adults with acquired severe aplastic anemia. *Bone Marrow Transplant*. (2019) 54:1319–26. doi: 10.1038/s41409-018-0410-3
74. Yang S, Yuan X, Ma R, Jiang L, Guo J, Zang Y, et al. Comparison of outcomes of frontline immunosuppressive therapy and frontline haploidentical hematopoietic stem cell transplantation for children with severe aplastic anemia who lack an HLA-matched sibling donor. *Biol Blood Marrow Transplant*. (2019) 25:975–80. doi: 10.1016/j.bbmt.2019.01.017
75. Zeng Y, Wang S, Wang J, Liu L, Su Y, Lu Z, et al. Optimal donor for severe aplastic anemia patient requiring allogeneic hematopoietic stem cell transplantation: a large-sample study from China. *Sci Rep*. (2018) 8:2479. doi: 10.1038/s41598-018-20853-9
76. Xu LP, Wang SQ, Wu DP, Wang JM, Gao SJ, Jiang M, et al. Haplo-identical transplantation for acquired severe aplastic anaemia in a multicentre prospective study. *Br J Haematol*. (2016) 175:265–74. doi: 10.1111/bjh.14225

77. Young NS. Aplastic anemia. *N Engl J Med*. (2018) 379:1643–56. doi: 10.1056/NEJMra1413485
78. Sun Q, Wu B, Lan H, Meng F, Ma X, Chen X, et al. Haploidentical haematopoietic stem cell transplantation for thalassaemia major based on an FBCA conditioning regimen. *Br J Haematol*. (2018) 182:554–8. doi: 10.1111/bjh.15438
79. Chen Y, Xu LP, Zhang XH, Chen H, Wang FR, Liu KY, et al. Busulfan, fludarabine, and cyclophosphamide (BFC) conditioning allowed stable engraftment after haplo-identical allogeneic stem cell transplantation in children with adrenoleukodystrophy and mucopolysaccharidosis. *Bone Marrow Transplant*. (2018) 53:770–3. doi: 10.1038/s41409-018-0175-8
80. Liu X, Wu M, Peng Y, Chen X, Sun J, Huang F, et al. Improvement in poor graft function after allogeneic hematopoietic stem cell transplantation upon administration of mesenchymal stem cells from third-party donors: a pilot prospective study. *Cell Transplant*. (2014) 23:1087–98. doi: 10.3727/096368912X661319
81. Kong Y, Chang YJ, Wang YZ, Chen YH, Han W, Wang Y, et al. Association of an impaired bone marrow microenvironment with secondary poor graft function after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2013) 19:1465–73. doi: 10.1016/j.bbmt.2013.07.014
82. Kong Y, Wang YT, Cao XN, Song Y, Chen YH, Sun YQ, et al. Aberrant T cell responses in the bone marrow microenvironment of patients with poor graft function after allogeneic hematopoietic stem cell transplantation. *J Transl Med*. (2017) 15:57. doi: 10.1186/s12967-017-1159-y
83. Kong Y, Wang YT, Hu Y, Han W, Chang YJ, Zhang XH, et al. The bone marrow microenvironment is similarly impaired in allogeneic hematopoietic stem cell transplantation patients with early and late poor graft function. *Bone Marrow Transplant*. (2016) 51:249–55. doi: 10.1038/bmt.2015.229
84. Zhao XY, Pei XY, Chang YJ, Yu XX, Xu LP, Wang Y, et al. First-line therapy with donor-derived HCMV-specific T cells reduces persistent HCMV infection by promoting antiviral immunity after allogenic stem cell transplantation. *Clin Infect Dis*. (2019) 2019:ciz368. doi: 10.1093/cid/ciz368
85. Ciurea SO, Thall PF, Milton DR, Barnes TH, Kongtim P, Carmazzi Y, et al. Complement-binding donor-specific anti-HLA antibodies and risk of primary graft failure in hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2015) 21:1392–8. doi: 10.1016/j.bbmt.2015.05.001
86. Chang YJ, Zhao XY, Xu LP, Zhang XH, Wang Y, Han W, et al. Donor-specific anti-human leukocyte antigen antibodies were associated with primary graft failure after unmanipulated haploidentical blood and marrow transplantation: a prospective study with randomly assigned training and validation sets. *J Hematol Oncol*. (2015) 8:84. doi: 10.1186/s13045-015-0182-9
87. Sun YQ, Wang Y, Zhang XH, Xu LP, Liu KY, Yan CH, et al. Virus reactivation and low dose of CD34+ cell, rather than haploidentical transplantation, were associated with secondary poor graft function within the first 100 days after allogeneic stem cell transplantation. *Ann Hematol*. (2019) 98:1877–83. doi: 10.1007/s00277-019-03715-w
88. Ciurea SO, Cao K, Fernandez-Vina M, Kongtim P, Malki MA, Fuchs E, et al. The European Society for Blood and Marrow Transplantation (EBMT) consensus guidelines for the detection and treatment of donor-specific anti-hla antibodies (DSA) in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant*. (2018) 53:521–34. doi: 10.1038/s41409-017-0062-8
89. Tang C, Chen F, Kong D, Ma Q, Dai H, Yin J, et al. Successful treatment of secondary poor graft function post allogeneic hematopoietic stem cell transplantation with eltrombopag. *J Hematol Oncol*. (2018) 11:103. doi: 10.1186/s13045-018-0649-6
90. Fu H, Zhang X, Han T, Mo X, Wang Y, Chen H, et al. Eltrombopag is an effective and safe therapy for refractory thrombocytopenia after haploidentical hematopoietic stem cell transplantation. *Bone Marrow Transplant*. (2019) 54:1310–8. doi: 10.1038/s41409-019-0435-2
91. Marotta S, Marano L, Ricci P, Cacace F, Frieri C, Simeone L, et al. Eltrombopag for post-transplant cytopenias due to poor graft function. *Bone Marrow Transplant*. (2019) 54:1346–53. doi: 10.1038/s41409-019-0442-3
92. Bramanti S, Calafiore V, Longhi E, Mariotti J, Crespiatico L, Sarina B, et al. Donor-specific anti-HLA antibodies in haploidentical stem cell transplantation with post-transplantation cyclophosphamide: risk of graft failure, poor graft function, and impact on outcomes. *Biol Blood Marrow Transplant*. (2019) 25:1395–1406. doi: 10.1016/j.bbmt.2019.02.020
93. Chen Y, Xu LP, Liu KY, Chen H, Chen YH, Zhang XH, et al. Risk factors for cytomegalovirus DNAemia following haploidentical stem cell transplantation and its association with host hepatitis B virus serostatus. *J Clin Virol*. (2016) 75:10–5. doi: 10.1016/j.jcv.2015.12.003
94. Zhao XS, Liu YR, Xu LP, Wang Y, Zhang XH, Chen H, et al. Minimal residual disease status determined by multiparametric flow cytometry pretransplantation predicts the outcome of patients with ALL receiving unmanipulated haploidentical allografts. *Am J Hematol*. (2019) 94:512–21. doi: 10.1002/ajh.25417
95. Liu J, Ma R, Liu YR, Xu LP, Zhang XH, Chen H, et al. The significance of peri-transplantation minimal residual disease assessed by multiparameter flow cytometry on outcomes for adult AML patients receiving haploidentical allografts. *Bone Marrow Transplant*. (2019) 54:567–77. doi: 10.1038/s41409-018-0300-8
96. Liu J, Zhao XS, Liu YR, Xu LP, Zhang XH, Chen H, et al. Association of persistent minimal residual disease with poor outcomes of patients with acute myeloid leukemia undergoing allogeneic hematopoietic stem cell transplantation. *Chin Med J*. (2018) 131:2808–16. doi: 10.4103/0366-6999.246072
97. Canaani J, Labopin M, Huang XJ, Ciceri F, Van Lint MT, Bruno B, et al. Minimal residual disease status predicts outcome of acute myeloid leukaemia patients undergoing T-cell replete haploidentical transplantation. An analysis from the Acute Leukaemia Working Party (ALWP) of the European Society for Blood and Marrow Transplantation (EBMT). *Br J Haematol*. (2018) 183:411–20. doi: 10.1111/bjh.15540
98. Zhao XS, Jin S, Zhu HH, Xu LP, Liu DH, Chen H, et al. Wilms' tumor gene 1 expression: an independent acute leukemia prognostic indicator following allogeneic hematopoietic SCT. *Bone Marrow Transplant*. (2012) 47:499–507. doi: 10.1038/bmt.2011.121
99. Zhao XS, Liu YR, Zhu HH, Xu LP, Liu DH, Liu KY, et al. Monitoring MRD with flow cytometry: an effective method to predict relapse for ALL patients after allogeneic hematopoietic stem cell transplantation. *Ann Hematol*. (2012) 91:183–92. doi: 10.1007/s00277-011-1285-1
100. Qin YZ, Chen Y, Xu LP, Wang Y, Zhang XH, Chen H, et al. Outcome and minimal residual disease monitoring in patients with t(16;21) acute myelogenous leukemia undergoing allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2018) 24:163–8. doi: 10.1016/j.bbmt.2017.09.002
101. Zhao X, Hong Y, Qin Y, Xu Y, Chang Y, Wang Y, et al. The clinical significance of monitoring the expression of the SIL-TAL1 fusion gene in T-cell acute lymphoblastic leukemia after allogeneic hematopoietic stem cell transplantation. *Int J Lab Hematol*. (2017) 39:613–9. doi: 10.1111/ijlh.12711
102. Wang Y, Wu DP, Liu QF, Qin YZ, Wang JB, Xu LP, et al. In adults with t(8;21)AML, posttransplant RUNX1/RUNX1T1-based MRD monitoring, rather than c-KIT mutations, allows further risk stratification. *Blood*. (2014) 124:1880–6. doi: 10.1182/blood-2014-03-563403
103. Tang FF, Xu LP, Zhang XH, Chen H, Chen YH, Han W, et al. Monitoring of post-transplant CBFB-MYH11 as minimal residual disease, rather than KIT mutations, can predict relapse after allogeneic haematopoietic cell transplantation in adults with inv(16) acute myeloid leukaemia. *Br J Haematol*. (2018) 180:448–51. doi: 10.1111/bjh.14340
104. Hong Y, Zhao X, Qin Y, Zhou S, Chang Y, Wang Y, et al. The prognostic role of E2A-PBX1 expression detected by real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) in B cell acute lymphoblastic leukemia after allogeneic hematopoietic stem cell transplantation. *Ann Hematol*. (2018) 97:1547–54. doi: 10.1007/s00277-018-3338-1
105. Liu J, Wang Y, Xu LP, Liu DH, Qin YZ, Chang YJ, et al. Monitoring mixed lineage leukemia expression may help identify patients with mixed lineage leukemia–rearranged acute leukemia who are at high risk of relapse after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2014) 20:929–36. doi: 10.1016/j.bbmt.2014.03.008
106. Zhu HH, Zhang XH, Qin YZ, Liu DH, Jiang H, Chen H, et al. MRD-directed risk stratification treatment may improve outcomes of t(8;21) AML in the first complete remission: results from the AML05 multicenter trial. *Blood*. (2013) 121:4056–62. doi: 10.1182/blood-2012-11-468348
107. Balsat M, Renneville A, Thomas X, de Botton S, Caillot D, Marceau A, et al. Postinduction minimal residual disease predicts outcome and

benefit from allogeneic stem cell transplantation in acute myeloid leukemia with NPM1 mutation: a study by the acute leukemia french association group. *J Clin Oncol*. (2017) 35:185–93. doi: 10.1200/JCO.2016.67.1875

108. Ciurea SO, Al Malki MM, Kongtim P, Fuchs EJ, Luznik L, Huang XJ, et al. The European Society for Blood and Marrow Transplantation (EBMT) consensus recommendations for donor selection in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant*. (2019). doi: 10.1038/s41409-019-0499-z. [Epub ahead of print].
109. Chang YJ, Wang Y, Liu YR, Xu LP, Zhang XH, Chen H, et al. Haploidentical allograft is superior to matched sibling donor allograft in eradicating pre-transplantation minimal residual disease of AML patients as determined by multiparameter flow cytometry: a retrospective and prospective analysis. *J Hematol Oncol*. (2017) 10:134. doi: 10.1186/s13045-017-0502-3
110. Chang YJ, Zhao XS, Wang Y, Liu YR, Xu LP, Zhang XH, et al. Effects of pre- and post-transplantation minimal residual disease on outcomes in pediatric patients with acute myeloid leukemia receiving human leukocyte antigen-matched or mismatched related donor allografts. *Am J Hematol*. (2017) 92:E659–61. doi: 10.1002/ajh.24910
111. Zhao X, Wang Z, Ruan G, Liu Y, Wang Y, Zhang X, et al. Impact of pre-transplantation minimal residual disease determined by multiparameter flow cytometry on the outcome of AML patients with FLT3-ITD after allogeneic stem cell transplantation. *Ann Hematol*. (2018) 97:967–75. doi: 10.1007/s00277-018-3265-1
112. Mariotti J, Devillier R, Bramanti S, Sarina B, Furst S, Granata A, et al. T cell-replete haploidentical transplantation with post-transplantation cyclophosphamide for hodgkin lymphoma relapsed after autologous transplantation: reduced incidence of relapse and of chronic graft-versus-host disease compared with HLA-identical related donors. *Biol Blood Marrow Transplant*. (2018) 24:627–32. doi: 10.1016/j.bbmt.2017.11.030
113. Gauthier J, Poiré X, Gac AC, Leclerc M, Guillaume T, Chalandon Y, et al. Better outcome with haploidentical over HLA-matched related donors in patients with Hodgkin's lymphoma undergoing allogeneic haematopoietic cell transplantation-a study by the Francophone Society of Bone Marrow Transplantation and Cellular Therapy. *Bone Marrow Transplant*. (2018) 53:400–9. doi: 10.1038/s41409-017-0018-z
114. Ringdén O, Labopin M, Ciceri F, Velardi A, Bacigalupo A, Arcese W, et al. Is there a stronger graft-versus-leukemia effect using HLA-haploidentical donors compared with HLA-identical siblings? *Leukemia*. (2016) 30:447–55. doi: 10.1038/leu.2015.232
115. Luo Y, Xiao H, Lai X, Shi J, Tan Y, He J, et al. T-cell-replete haploidentical HSCT with low-dose anti-T-lymphocyte globulin compared with matched sibling HSCT and unrelated HSCT. *Blood*. (2014) 124:2735–43. doi: 10.1182/blood-2014-04-571570
116. Fleischhauer K, Hsu KC, Shaw BE. Prevention of relapse after allogeneic hematopoietic cell transplantation by donor and cell source selection. *Bone Marrow Transplant*. (2018) 53:1498–507. doi: 10.1038/s41409-018-0218-1
117. Cooley S, Parham P, Miller JS. Strategies to activate NK cells to prevent relapse and induce remission following hematopoietic stem cell transplantation. *Blood*. (2018) 131:1053–62. doi: 10.1182/blood-2017-08-752170
118. Mo XD, Tang BL, Zhang XH, Zheng CC, Xu LP, Zhu XY, et al. Comparison of outcomes after umbilical cord blood and unmanipulated haploidentical hematopoietic stem cell transplantation in children with high-risk acute lymphoblastic leukemia. *Int J Cancer*. (2016) 139:2106–15. doi: 10.1002/ijc.30249
119. Wang Y, Chen H, Chen J, Han M, Hu J, Jiong Hu, et al. The consensus on the monitoring, treatment, and prevention of leukemia relapse after allogeneic hematopoietic stem cell transplantation in China. *Cancer Lett*. (2018) 438:63–75. doi: 10.1016/j.canlet.2018.08.030
120. Stein AS, Kantarjian H, Gökbuget N, Bargou R, Litzow MR, Rambaldi A, et al. Blinatumomab for acute lymphoblastic leukemia relapse after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2019) 25:1498–504. doi: 10.1016/j.bbmt.2019.04.010
121. Jen EY, Xu Q, Schetter A, Przepiorka D, Shen YL, Roscoe D, et al. FDA approval: blinatumomab for patients with B-cell precursor acute lymphoblastic leukemia in morphologic remission with minimal residual disease. *Clin Cancer Res*. (2019) 25:473–7. doi: 10.1158/1078-0432.CCR-18-2337
122. Gökbuget N, Dombret H, Bonifacio M, Reichle A, Graux C, Faul C, et al. Blinatumomab for minimal residual disease in adults with B-cell precursor acute lymphoblastic leukemia. *Blood*. (2018) 131:1522–31. doi: 10.1182/blood-2017-08-798322
123. Tan Y, Du K, Luo Y, Shi J, Cao L, Zheng Y, et al. Superiority of preemptive donor lymphocyte infusion based on minimal residual disease in acute leukemia patients after allogeneic hematopoietic stem cell transplantation. *Transfusion*. (2014) 54:1493–500. doi: 10.1111/trf.12524
124. Brodsky RA, Luznik L, Bolaños-Meade J, Leffell MS, Jones RJ, Fuchs EJ. Reduced intensity HLA-haploidentical BMT with post transplantation cyclophosphamide in nonmalignant hematologic diseases. *Bone Marrow Transplant*. (2008) 42:523–7. doi: 10.1038/bmt.2008.203
125. Solomon SR, Sizemore CA, Sanacore M, Zhang X, Brown S, Holland HK, et al. Haploidentical transplantation using T cell replete peripheral blood stem cells and myeloablative conditioning in patients with high-risk hematologic malignancies who lack conventional donors is well tolerated and produces excellent relapse-free survival: results of a prospective phase II trial. *Biol Blood Marrow Transplant*. (2012) 18:1859–66. doi: 10.1016/j.bbmt.2012.06.019
126. Yu X, Liu L, Xie Z, Dong C, Zhao L, Zhang J, et al. Bone marrow versus peripheral blood as a graft source for haploidentical donor transplantation in adults using post-transplant cyclophosphamide-A systematic review and meta-analysis. *Crit Rev Oncol Hematol*. (2019) 133:120–8. doi: 10.1016/j.critrevonc.2018.05.017

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The Evolution of T Cell Depleted Haploidentical Transplantation

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Work on bone marrow transplantation from haploidentical donor has been proceeding for over 20 years all over the world and new transplant procedures have been developed. To control both graft rejection and graft vs. host disease, some centers have preferred to enhance the intensity of the conditioning regimens and the post-transplant immune suppression in the absence of graft manipulation; others have concentrated on manipulating the graft in the absence of any additional post-transplant immune suppressive agent. Due to the current high engraftment rates, the low incidence of graft-vs.-host disease and regimen related mortality, transplantation from haploidentical donors have been progressively offered even to elderly patients. Overall, survivals compare favorably with reports on transplants from unrelated donors. Further improvements will come with successful implementation of strategies to enhance post-transplant immune reconstitution and to prevent leukemia relapse.

Keywords: haploidentical transplantation, T cell depletion, graft vs. host disease, immune reconstitution, graft vs. leukemia effect

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INTRODUCTION

The great interest in hematopoietic stem cell transplantation (HSCT) from partially matched family donors (haplo-HSCT) arises from several advantages: (1) the donor is immediately available for almost all patients, (2) he/she can be chosen from family members, (3) he/she is highly motivated, and (4) post-transplant donor-derived cellular therapies (such as donor lymphocyte infusions) are easily accessible if needed (1). A recent survey confirmed the numbers of haplo-HSCT performed in Europe continue to increase (2), certainly because of the impressive progress in the clinical, biological, and technical aspects of haplo-HSCT that have been achieved over the past decade. Nowadays, haplo-HSCT is a clinical reality that provides similar outcomes to transplantation from either matched unrelated donors or unrelated cord blood unit (3–5).

Approaches to T-cell depletion have varied greatly in the levels of residual T lymphocytes in the inoculum, intensity of the conditioning regimens and post-transplant immunosuppression (6). On the other hand, interest in unmanipulated or T cell replete haplo-HSCT was reawakened by new strategies for graft-vs.-host disease (GvHD) prophylaxis, such as G-CSF–primed grafts (7, 8), post-transplant rapamycin (9), or high-dose cyclophosphamide (CY) in combination with other immunosuppressive agents (10–13). This latter is mainly based on standard T cell replete stem cell transplants with the aim of making haplo-HSCT as easier as possible. On the contrary, transplant platforms based on T cell depletion rely mainly on graft processing to achieve an ideal graft composition that allows for prevention of rejection, recurrence of leukemia, infections, and GvHD without the need for any further post-transplant immune suppressive

treatment. T cell depletion-based grafts require dedicated laboratories and are more expensive than conventional unmanipulated HSCT, especially if combined with adoptive transfer of T cell populations that have been chosen to improve post-transplant immune reconstitution. However, unlike in unmanipulated haplo-HSCT, pharmacologic GvHD prophylaxis and further treatments are not necessary in T cell depleted haplo-HSCT, thus, reducing the need and the cost of supportive care and post-transplant hospitalizations. In recipients of unmanipulated T replete haplo-HSCT followed by post-transplant cyclophosphamide (PTCY), the use of non-ablative conditioning regimen has certainly contributed to reduce non-relapse mortality (NRM). However, non-fatal BK virus-associated hemorrhagic cystitis (HC) occurred in 75% of patients after a busulfan-based conditioning and in 30% of patients after a TBI-based conditioning [reviewed in (14)]. Furthermore, relapse remains a major concern, especially after non-myeloablative conditioning regimens, occurring in ∼45–51% of patients (11, 14, 15).

This review will concentrate on the evolution of the T cell depleted (TCD) haplo-HSCT since the main obstacles to its success (lethal GvHD and graft rejection) were overcome in the early 1990s.

PREVENTING GvHD AND OVERCOMING REJECTION

In the early 1980s the use of soybean lectin agglutination (SBA) followed by rosetting with sheep red blood cells (E-rosette) allowed hematopoietic stem cell engraftment and immune reconstitution in the absence of GvHD. The sedimentation of T cells that spontaneously surrounded sheep red cells (rosettes) made possible the depletion of most of the T lymphocytes that escaped lectin agglutination. Such approach resulted in about a thousand-fold depletion of T-cells (16, 17). Thanks to this technique, patients with severe combined immunodeficiency (SCID) were successfully transplanted with TCD bone marrow graft from a haploidentical donor. T cell depletion facilitated engraftment and ensured no GvHD in these patients (16, 17).

Since the first successful haplo-HSCT in SCID patients, a lectin-based T cell depletion approach has been implemented over the years with great success in hundreds of SCID patients with a very long follow-up. It showed a cure rate that was especially impressive in patients receiving the transplant within the first year after birth (16–19). Following these remarkable results, TCD haplo-HSCT was attempted in patients affected by acute leukemias. In the first patient series such approach failed because of an unacceptable high rate of graft rejection (20, 21). In leukemia patients, anti-donor, recipient type, cytotoxic T-lymphocyte precursors (CTL-p) may survive the conditioning regimen and promote rejection of the donor graft (21–23). Donor T cells that derives from the inoculum eliminate residual host CTL-ps and allow for engraftment in unmanipulated transplants. Such mechanism is not present in TCD transplants. Thus, conditioning regimens that might be conventionally considered sufficient for donor engraftment in unmanipulated transplants are no longer adequate in TCD haplo-HSCT.

The use of a graft containing a "megadose" of TCD hematopoietic progenitor cells was a clinical breakthrough as it overcame such immunological barrier in the absence of an excessive conditioning regimen related toxicity (24, 25). Preclinical studies demonstrated that cells within the human CD34+ hematopoietic stem cell population can specifically neutralize CTL-ps directed against their antigens but not against a third party in mixed lymphocyte reactions. This peculiar ability was called "veto" activity (26–28). The ability of a "megadose" of CD34+ cells to exert *in vivo* "veto" activity and, thus, to facilitate engraftment, was confirmed in a "first in human" clinical trial in Perugia from 1993 to 1995. In this study, TCD haplo-HSCT was performed in 36 acute leukemia patients that received a conditioning regimen with single dose total body irradiation (TBI), cyclophosphamide, anti-thymocyte globulin (ATG), and thiotepa followed by the infusion of $\approx 10 \times 10^6$ CD34+ cells/kg and only 2×10^5 CD3+ cells/kg. This clinical protocol showed robust sustained engraftment in 80% of patients with only 20% of them experiencing GvHD despite the absence of any pharmacologic immune suppressive GvHD prophylaxis (29).

FROM LECTINS TO CD34+ CELL SELECTION

Following this initial success, efforts have been made to optimize graft processing and reducing the conditioning-related toxicity with the aim to further improve TCD haplo-HSCT outcome.

Grafts containing a median of 2×10^5 CD3+ cells/kg after the lectin-based procedure were associated to a 20% incidence of GvHD. Moreover, in SCID haplo-HSCT, 3×10^4/kg of donor T cells was identified as the threshold for GvHD (17). To further reduce the number of T lymphocytes in the final graft to such level, peripheral blood progenitor cells (PBPCs) mobilized with G-CSF were depleted of T-cells by one round of E-rosetting followed by positive immuno-selection of the CD34+ cells with the Ceprate system (30). This strategy was subsequently abandoned in 1999 when the CliniMACS device (©Miltenyi) allowed for an effective CD34+ cell selection in just one step procedure. This approach is still widely used to date as no other manipulation of leukapheresis products is needed (31).

In 1995 the Perugia group started to use fludarabine instead of cyclophosphamide for the first time in allogeneic HSCT. This modification of the conditioning regimen was based on data from a murine model where conditioning regimens with TBI/cyclophosphamide and TBI/fludarabine provided similar immunosuppression (32). In fact, fludarabine was introduced in order to minimize extra-hematological toxicity and, at the same time, to enhance host immunosuppression (30, 31).

The combination of a fludarabine-based conditioning regimen and the positive selection of the CD34+ cells prevented both rejection and GvHD. However, it is worth noting that *in vivo* persistence of ATG, which was part of the conditioning, may have contributed to the almost complete control of GvHD. At the same time, the conditioning-related toxicity was very low

with only a minority of patients developing severe mucositis and no case of veno-occlusive disease of the liver was observed (31).

An analysis of the relapse rate also led to some interesting observations. In fact, despite the absence of GvHD, the leukemia relapse was not increased in these high-risk leukemia patients (31). Several factors may have contributed to eradicate the residual leukemic cells despite the lack of a potent T-cell mediated Graft-vs.-Leukemia (GvL) effect: (1) the intense myeloablation of the conditioning regimen could have reached a deeper reduction of leukemic stem cells in the bone marrow of the patients; (2) the few T cells in the graft may have exerted a subclinical GvL/GvHD effect because they were unopposed by any post-transplant immune suppressive treatment; (3) a strong and T cell independent GvL effect exerted by donor NK cells (33–35). NK-cell function is regulated by a balance of signals mediated by activating and inhibitory receptors (36). NK receptors specific for major histocompatibility complex (MHC) class I molecules, including killer immunoglobulin (Ig)-like receptors (KIR) and the C-type lectin-like CD94/NKG2A, have a role in eradicating residual leukemic cells. NK cells react to the lack of self-HLA expression on allogeneic targets (so-called "missing self-recognition") (37). In an analysis of 112 patients with high-risk AML, transplantation from NK-alloreactive donors ($n = 51$) was associated with a significantly lower relapse rate in the 61 patients in complete remission (CR) at transplant (3 vs. 47%) ($P > 0.003$) and better event-free survival (EFS) (67 vs. 18%, $P = 0.02$) (38). Results from clinical trials have shown that NK cell alloreactivity is also an effective form of immunotherapy in pediatric acute leukemia (39, 40). The combination of KIR genes define group A haplotype, which has few genes, most of which encoding for inhibitory KIRs, while group B, in addition to inhibitory KIRs, has several genes encoding for activating KIRs (40). In children with acute lymphoid leukemia in complete remission, Oevermann et al. reported a significantly reduced incidence of relapse among the group B haplotype as compared to those of the group A haplotype (33 vs. 64%) (41). Another mechanism that allows for better control of leukemia relapse relies on the use of mothers as donors. In fact, mothers can develop memory T cells against paternal HLA haplotype because of exposure to fetal antigens during pregnancy. This T cell immunity could be responsible for early recognition of such antigens in leukemic cells after transplant resulting in stronger GVL effect when mothers are chosen as donors (42). In addition to the anti-leukemia effect, NK-alloreactive donors carrying KIR2DS1 and/or KIR3DS1 genes also impact on NRM by controlling infections, and so contributing to improve the event-free survival (43). Therefore, the donor-vs.-recipient NK alloreactivity, as predicted by the HLA disparity, should be considered when selecting the optimal donor within the family members.

Apart from the NK alloreactivity, 43% of AML and 30% of ALL patients who were in any CR at transplant survive event-free and GvHD-free with a maximum follow-up of 20 years (31). More recently, the European Group for Blood and Marrow Transplantation (EBMT) performed a retrospective study collecting data from different European centers to analyze the outcome of "mega-dose" haplo-HSCT. This study confirmed the success of the approach by reporting 48% EFS in 266 patients with AML in first CR at the time of transplantation (44).

POSITIVE SELECTION OF THE PBPCs AND POST-TRANPLANT IMMUNOLOGICAL RECONSTITUTION

While the low number of infused donor T lymphocytes allows for almost full prevention of GvHD in TCD haplo-HSCT, it is also responsible for the major drawback of this approach. In fact, post-transplant T cell immune reconstitution in TCD haplo-HSCT is delayed because it relies only on the expansion of the few T cells infused within the graft and on the development of donor, thymus derived, naïve T cells that occurs several months after transplant in adult patients. Thymus function decays with age and myeloablative conditioning regimen further disrupts thymus and lymphoid structures. These events alter post-transplant T cell dynamics and impede generation of efficient memory T cell immunity (45). Because of the low number of donor T cell in the graft and the additional *in vivo* T cell depletion exerted by the use of ATG in the conditioning regimen, patients that receive TCD haplo-HSCT exhibit a very narrow T-cell repertoire that is responsible for their prolonged susceptibility to life-threatening opportunistic infections (46). In the study by Aversa and colleagues, 27 of 103 patients died because of deadly infections (31). Thus, infection-related mortality was the main cause of transplant failure in this setting.

In this context, it is of note the retrospective analysis that the Swiss Blood Stem Cell Transplantation group made to evaluate the effect on immune reconstitution and incidence of infections in haplo-HSCT from 1998 to 2010. The authors reported 69 transplants that were performed with *ex-vivo* T cell depletion (through CD34 positive selection or CD3/CD19 depletion) or with *in vivo* T cell depletion using anti-CD52 monoclonal antibody alemtuzumab (47). High incidence of life-threatening bacterial, fungal, and viral infections (mostly Cytomegalovirus, CMV) was reported in all these patients. Eventually, the use of alemtuzumab was associated with a higher incidence of CMV reactivations (54 vs. 28%, $p = 0.015$), demonstrating that even *in vivo* T cell depletion should be considered a relevant risk factor in haplo-HSCT (48).

Improving Immunological Reconstitution After CD34+ Cell Haplo-HSCT

With the aim of diminishing the challenges of life-threatening infections, GvHD, and relapse after TCD haplo-HSCT, various strategies have been investigated over the past decade to facilitate the safe transfer of mismatched T lymphocytes.

The use of post-transplant adoptive transfer of pathogen-specific T lymphocytes represents a possible strategy. A study demonstrated that the infusion of donor derived *ex vivo* selected T cells that were able to clone specifically against Aspergillus or CMV antigens, could control CMV reactivation and reduced detection of galactomannan (49). Interestingly these cells remained pathogen-specific over time after infusion as patients did not develop such infections and did not

experience GvHD. Thanks to these promising results, other authors developed similar approaches for the prevention of Adenovirus and Epstein-Barr virus (EBV) infections (50, 51).

Several groups attempted to ameliorate post-transplant immune reconstitution by infusing adoptive T cell immunotherapy with a broad T cell receptor repertoire that resembles physiological conditions. Different approaches have been used to manipulate the graft so that enough T cells could be infused to the patients without causing GvHD.

The group form San Raffaele Institute in Milan, Italy, engineered polyclonal donor T cells to express suicide genes (e.g., the herpes simplex thymidine kinase, TK, and gene). Once infused, these engineered cells could be lysed in case they triggered GvHD by the simple use of Ganciclovir, a widely available anti-viral kinase drug normally used in the treatment of CMV reactivations or diseases (52). One concern is that the mechanism is dependent on cell cycle, thus killing can be delayed and is limited to proliferating cells. Nevertheless, the patients enrolled in this study experienced a low rate of infection-related mortality suggesting functional protection against pathogens (53, 54). Thanks to this experience, it was further possible to understand that TK cell dependent immune reconstitution relies on the thymic generation of T cells derived from differentiated donor hematopoietic precursors (55).

An alternative and more attractive approach is based on the post-transplant infusion of inducible human caspase-9 transgene (iC9) T lymphocytes (56, 57). This technology is based on a cell membrane-permeable small molecule dimerizing drug, AP1903 (also known as Rimiducid). The administration of AP1903 induces dimerization of caspase 9, which activates the terminal effector caspase, caspase 3, with rapid induction of apoptosis. Unlike the HSV-TK–based suicide gene, the iC9 is human derived and has limited immunogenicity and, more important, ganciclovir and related drugs to treat viral infection are allowed without T-cell damage (56). Activation of iC9 produces up to 99% eradication of iC9-expressing T cells within 2 h of a single dose of AP1903 and controls GvHD within 24–48 h. Although administration of AP1903 in patients with GvHD reduces the level of circulating virus-specific iC9-T cells, these cells subsequently recover and *in vivo* antiviral activity is retained.

Another approach aims to *ex vivo* selectively deplete donor-vs.-recipient alloreactive T lymphocytes. T-cell activation is associated with P-glycoprotein pump inhibition, which leads to intracellular accumulation of the rhodamine-derived photosensitizer TH9402, a substrate of this pump Alloreactive T cells preferentially retain the photosensitizer TH9402 and can then be eliminated following exposure to visible light. On the contrary, resting T lymphocytes still exhibit a broad repertoire against infective agents (58–60). More recently, this photodepletion strategy has been tested in a phase I clinical study with aims to find the maximum tolerated dose and to evaluate the safety of allodepleted T-cell immunotherapy (ATIR101), administered in the absence of any additional GvHD prophylaxis, in recipients of CD34+-selected haploidentical HSCT (61). Adults with hematological malignancies were treated with myeloablative TCD haplo-HSCT followed 1 month later by ATIR101 at escalating doses. No patient developed grade III/IV acute GVHD. At 1 year, all nine patients receiving at least one million ATIR101 CD3+ cells/kg did not experience life-threatening infections. After more than 8 years, none of them died because of non-relapse mortality and two thirds of them survive. These promising results set the base for the development of an ongoing phase 3 randomized trial that compares haplo-HSCT + ATIR101 vs. unmanipulated haplo-HSCT + PTCY.

Recently, the group of Perugia employed adoptive transfer of donor CD4+CD25+FOXP3+ regulatory T cells (Tregs) to protect from GvHD that could be caused by the concomitant infusion of high numbers of donor conventional T cells (Tcons) (62, 63). Freshly isolated donor Tregs at a dose of 2×10^6 /kg were given 4 days prior to the infusion of a "megadose" of CD34-positive cells and controlled numbers ($0.5–2 \times 10^6$/kg) of broad repertoire Tcons, without any post-transplant immunosuppression. GvHD occurred in a minority of the patients proving the effectiveness of the approach despite no post-transplant pharmacologic immune suppressive drug was given to the patients. Moreover, Treg/Tcon adoptive immunotherapy allowed for a fast post-transplant T and B cell immune reconstitution. Diverse naïve and memory T cell subpopulations with a broad T cell receptor repertoire could be early detected and rapidly increased over time. Pathogen-specific CD4+ and CD8+ T cell clones emerged earlier in comparison to patients that received TCD haplo-HSCT with no Treg/Tcon infusions. Treg/Tcon adoptive immunotherapy reduced CMV reactivation episodes with no CMV-related death. More importantly, Treg infusion did not interfere with Tcon mediated GvL effect as leukemia relapse occurred in very few patients despite high-risk diseases (64).

FROM POSITIVE TO NEGATIVE SELECTION OF PBPCs

More recently, the CD34-positive selection technique has been progressively abandoned in favor of a negative selection of the PBPCs with the aim of improving clinical results. In fact, unlike the CD34-positive selected grafts, other immune components, such as NK cells, dendritic cells, and monocytes, are not lost during the negative selection-based procedure and all together these cells contribute to facilitate engraftment, to improve the post-transplant recovery of the anti-infective and anti-leukemia immunity.

Negative Selection in Children

In the study by Bader et al. (65), grafts were depleted of T and B cells by using CD3- and CD19-coated microbeads and the automated CliniMACS device (Miltenyi Biotec, Germany). Children with acute leukemia received a conditioning that included fludarabine, thiotepa, melphalan, and OKT-3 or ATG. Primary engraftment was achieved in 88% of patients, acute GvHD grade II and III-IV occurred in 20 and 7%, and chronic GvHD in 21%. NRM was 8% at 1 year and 20% at 5 years (65). Using the same T and B cell depletion but a reduced intensity conditioning in 61 adults (median age 46 years), the

incidence of grade II–IV acute and chronic GvHD was 46 and 18%, respectively. Non-relapse mortality on Day 100 was 23 and 42% at 2 years. Relapse rate was 31% and OS at 2 years was 28% (66). A major concern with this approach was the high incidence of GvHD.

To overcome this problem, Chaleff et al. recently described a large-scale clinical method using the Miltenyi Biotec CliniMACS® TCR α/β System for the depletion of α/β T lymphocytes from peripheral blood stem cells while retaining all other cells (67). The CliniMACS® TCR α/β System uses murine monoclonal antibodies specific for the T-cell receptor α/β antigen conjugated to biotin in combination with the CliniMACS® Anti-Biotin reagent. The pioneering experience of the Handgretinger's group showed that TCR αβ/CD19 depletion allows a T-cell reduction of 4.5–5 log, which is comparable to CD34+ positive selection (68, 69). It also ensures patients to receive NK cells, monocytes, dendritic cells, and, most important, the TCRγδ+ T lymphocytes. TCRγδ+ T cells appear to exert anti-leukemic activity since they directly recognized stress-induced self-antigens expressed by malignant cells. Strikingly, they do not recognize specific processed peptide antigens as presented on major histocompatibility complex molecules and so are not expected to induce GvHD (70–72).

The first clinical experiences with children transplanted in Tubingen confirmed excellent full-donor engraftment, a rapid early expansion of donor-derived TCRγδ+ T lymphocytes that contributed to a very fast immunological reconstitution (69). Using the same method for graft processing, Locatelli and colleagues in Rome achieved similar results in terms of engraftment, prevention of both acute and chronic GvHD and a rapid recovery of post-transplant immunity in children independently from the conditioning regimen, whether TBI-based (children with leukemia) or chemotherapy-based (children with non-malignant disorders) (73, 74). In 23 children with non-malignant disorders, no cases of visceral acute or chronic GvHD was observed and survival was 91% at 2 years (75). The same group in Rome, starting from encouraging results on a chimeric gene incorporating the death domain of inducible caspase 9 (iC9) (56, 76), has recently launched a phase I/II study enrolling children with either malignant or non-malignant disorders who will receive TCRαβ/CD19-depleted haplo-HSCT, followed by the infusion of titrated numbers of iC9 T cells on day 14 ± 4. These iC9-modified T cells are expected to further improve T cell immune reconstitution without the risk of severe GvHD. In fact, they can be rapidly eliminated by the administration of AP1903, if acute GvHD occurs (77).

TCRγδ+ T cell recovering during the first year after HSCT in 102 patients with acute leukemia correlated with a reduced incidence of infection in the study by Perko et al. (78). Children with an elevated number of TCRγδ+ T cells post-engraftment experienced only viral infection, while low/normal TCRγδ+ T cell group had viral, bacterial and fungal infections. Enhanced TCRγδ+ T cell recovery resulted also in higher EFS rate at 1 year. One can speculate that the following factors may contribute to explain these excellent results: a very fast reconstitution of intestinal mucosa integrity, prompt anti-infective function of TCRγδ+ T cell, and possibly a better balance within gut microbiota (79).

Outcomes of TCRαβ/CD19-depleted haplo-HSCT were evaluated in a cohort of children with chemorefractory AML. The conditioning regimen was designed to include a cytoreduction phase with fludarabine and cytarabine followed by a myeloablative phase with treosulfan and thiotepa. Tocilizumab was given instead ATG in all patients, abatacept in 10 patients. Post-engraftment CD45RA-depleted donor lymphocytes were given prophylactic with or without a hypomethylating agent. Overall results were promising with 95% of patients achieving a complete remission, 18% having a grade II-IV acute GvHD and 23% chronic GvHD. At 2 years, NRM was 9%, relapse rate 42%, event-free and overall survival were 49 and 53%, respectively (80).

More recently, the advantages of this strategy were confirmed in 20 advanced-stage Sickle Cell Disease (SCD) patients (children and adults, median age 15 years). Conditioning consisted of ATG, thiotepa, fludarabine, and treosulfan. Two patients succumbed to a CMV pneumonitis and a macrophage activation syndrome. One patient requires renal replacement therapy because of BK virus nephritis. None developed grade III-IV acute GvHD. At a median follow-up of 21 (range 9–62) months, 90% of these high-risk patients survive showing the feasibility, safety, and efficacy of TCD haplo-HSCT also for advanced stage SCD patients (81).

Negative Selection in Adults

This approach was recently tested in 59 adult patients (median age 48 years, range 19–74) with hematological malignancies, mostly acute leukemias (82). At the time of transplant, 35 (60%) were in first or later remission and 24 (40%) in advanced phase. All patients were conditioned with a chemotherapy-based regimen that included ATG, treosulfan, fludarabine, and thiotepa. No additional pharmacologic prophylaxis for GvHD was given after transplantation and to minimize the *in-vivo* T cell depletion, ATG was given at a median of 10 days before the graft infusion. A full donor sustained engraftment was achieved in 56/59 (95%) patients. Severe GvHD occurred in two patients who subsequently died from complications due to the GvHD itself and its treatment. One of them had received the highest dose of αβ+ T cells (3.7×10^5/kg). Skin limited grade II acute GvHD was observed in 8 patients who responded rapidly to steroids. Only two patients have so far developed chronic GvHD that recovered completely after steroid and cyclosporine treatment. Interestingly, also in these adults with high-risk hematological malignancies, numbers and functions of the immune system recovered very soon after the engraftment. Naïve and memory T-cell subsets increased significantly over the first year after transplantation. B-cell reconstitution was rapid and immunoglobulin serum levels normalized within 3 months. The quality of the immunological reconstitution allowed a good control of the CMV reactivation with no cases occurring after the first 2 months after transplantation. In two patients, CMV reactivation was associated with a significant expansion of pathogen-specific CD8+ T cells that contributed to clear viral load spontaneously. Relapse of the underlying disease was the main cause of death in 16/59 patients; 15 patients died

TABLE 1 | Evolution of T cell depleted allogeneic HSCT.

Years	Technique	Donor	Disease
1980s	TCD BM cells by SBA+E-rosetting	Matched Haplo	HMs SCID
1990s	TCD PBPCs by positive selection of the CD34+ cells using the CliniMACS device	Matched Haplo	HMs, N-MHs HMs, N-MHs
Early 2000s	CD3/CD19 selection of the PBPCs	Haplo	HMs, N-MHs
Late 2000s	CD34+ cell selection + Tregs/Tcons	Haplo	HMs
Late 2000s	Selective depletion of TCRαβ/CD19 cells in some center followed by cell therapy with HSTK-engineered lymphocytes, photodynamic purged T cells, iC9	Matched Haplo	HMs, N-MHs HMs, N-MHs

TCD, T Cell Depleted; BM, Bone Marrow; SBA, SoyBean Agglutinin; PBPCs, Peripheral Blood Progenitor Cells; Tregs, T regulatory cells; Tcons, Conventional T cells; HMs, Hematological Malignancies; N-MHs, Non-Malignant Hematologic diseases; HSTK, Herpes Simplex Thymidine Kinase; iC9, inducible Caspase-9.

without relapsing, 11 of them from infections. Age at time of transplantation was a significant risk factor for NRM. Three of the 28 patients (10.7%) aged ≤48 years and 12 of the 31 (38.7%) over 48 years of age have so far died from non-relapsing causes. Cumulative incidence of NRM at 2 years was 18% for patients aged ≤48 years and 47% for those over 48 years of age ($p = 0.011$). At a median of 27 months (range 1–62), 30 patients (50.8%) survive.

Similar results have been recently reported by a team in Turkey in 34 adult patients with either AML ($n = 24$) or ALL ($n = 10$) (83). Conditioning regimen consisted of thiotepa, melphalan, fludarabine, and ATG. Full donor chimerism was achieved in 31/34 patients. Overall, four patients developed severe GvHD (2 acute, 2 chronic). A low NRM (11.7%) at day 100 was attributed to a rapid T-cell reconstitution. Relapse still remained the main cause of death (56.3%). At 1 year, 42% of the patients survive disease-free.

CONCLUSIONS

Haplo-HSCT is an attractive treatment for patients with high-risk hematological malignancies lacking a well-matched unrelated donor and who require a HSCT urgently. Today, rejection and GvHD are no longer major issues and a recent registry-based study of the EBMT confirmed that outcomes of TCD haplo-HSCT have improved over time reflecting gaining experience, better selection of the donor-recipient pairs, evolution of the conditioning regimens, better supportive care, and treatment options for infections complications, that remain the main cause of death in this setting (84).

Years of research have taken us from a haplo-HSCT containing a megadose of CD34-positive cells and very few donor T lymphocytes to a new "designed" graft containing a megadose of selectively depleted PBPCs and also different types of non-alloreactive immune cells meant to improve immune recovery in the absence of any additional post-transplant immune suppressive prophylaxis of the GvHD (**Table 1**).

In this way, an innovative strategy has been recently designed by the Perugia group using a Total Marrow/Total Lymphoid Irradiation-based conditioning regimen followed by the infusion of TCD Treg/Tcon haplo-HSCT to treat elderly patients (aged 55–68 years) with acute myeloid leukemia. None of the first 14 transplanted patients have so far relapsed (85). On the other hand, in a recent retrospective cohort study by Solomon et al., recurrent disease was the main cause of death, in particular in patients aged 55–70 years for whom a RIC protocol was adopted to minimize the transplant-related toxicity (86).

In conclusion, we believe that TCD is still valid in haplo-HSCT for the following main reasons: (a) it guarantees patients to have a good quality of life in the absence of GvHD, in particular in the elderly who, due to the age-related comorbidities, are less able to tolerate GvHD and its treatments; (b) it provides a safer platform for advanced treatment with infusions of TCR-transgenic T-cells, genetically modified redirected NK cells or donor T cells bearing chimeric antigen receptor (CAR-T) to reduce, or even abrogate, the risk of recurrence of the underlying disease.

AUTHOR CONTRIBUTIONS

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REFERENCES

1. Apperley J, Niederwieser D, Huang XJ, Nagler A, Fuchs E, Szer J, et al. Haploidentical hematopoietic stem cell transplantation: a global overview comparing Asia, the European Union, and the United States. *Biol Blood Marrow Transplant*. (2016) 22(3 Suppl):S15–8. doi: 10.1016/j.bbmt.2016.01.006
2. Passweg JR, Baldomero H, Bader P, Bonini C, Duarte RF, Dufour C, et al. Use of haploidentical stem cell transplantation continues to increase: the 2015 European Society for Blood and Marrow Transplant activity

survey report. *Bone Marrow Transplant.* (2017) 52:811–7. doi: 10.1038/bmt.2017.34
3. Sun Y, Beohou E, Labopin M, Volin L, Milpied N, Yakoub-Agha I, et al. Unmanipulated haploidentical versus matched unrelated donor allogeneic stem cell transplantation in adult patients with acute myelogenous leukemia in first remission: a retrospective pair-matched comparative study of the Beijing approach with the EBMT database. *Haematologica*. (2016) 101:e352–4. doi: 10.3324/haematol.2015.140509
4. Bertaina A, Zecca M, Buldini B, Sacchi N, Algeri M, Saglio F, et al. Unrelated donor vs HLA-haploidentical α/β T-cell- and B-cell-depleted HSCT in children with acute leukemia. *Blood*. (2018) 132:2594–607. doi: 10.1182/blood-2018-07-861575
5. Ruggeri A, Labopin M, Sanz G, Piemontese S, Arcese W, Bacigalupo A, et al. Comparison of outcomes after unrelated cord blood and unmanipulated haploidentical stem cell transplantation in adults with acute leukemia. *Leukemia*. (2015) 29:1891–900. doi: 10.1038/leu.2015.98
6. Martelli MF, Aversa F. Haploidentical transplants using *ex vivo* T-cell depletion. *Semin Hematol.* (2016) 53:252–6. doi: 10.1053/j.seminhematol.2016.07.001
7. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W, et al. Treatment of acute leukemia with unmanipulated HLA-mismatched/haploidentical blood and bone marrow transplantation. *Biol Blood Marrow Transplant*. (2009) 15:257–65. doi: 10.1016/j.bbmt.2008.11.025
8. Di Bartolomeo P, Santarone S, De Angelis G, Picardi A, Cudillo L, Cerretti R, et al. Haploidentical, unmanipulated, G-CSF-primed bone marrow transplantation for patients with high-risk hematologic malignancies. *Blood*. (2013) 121:849–57. doi: 10.1182/blood-2012-08-453399
9. Peccatori J, Forcina A, Clerici D, Crocchiolo R, Vago L, Stanghellini MT, et al. Sirolimus-based graft-versus-host disease prophylaxis promotes the *in vivo* expansion of regulatory T cells and permits peripheral blood stem cell transplantation from haploidentical donors. *Leukemia*. (2015) 29:396–405. doi: 10.1038/leu.2014.180
10. Fuchs EJ. Human leukocyte antigen-haploidentical stem cell transplantation using T-cell-replete bone marrow grafts. *Curr Opin Hematol*. (2012) 19:440–7. doi: 10.1097/MOH.0b013e32835822dc
11. Sugita J. HLA-haploidentical stem cell transplantation using posttransplant cyclophosphamide. *Int J Hematol*. (2019) 110:30–8. doi: 10.1007/s12185-019-02660-8
12. Raiola AM, Dominietto A, Ghiso A, Di Grazia C, Lamparelli T, Gualandi F, et al. Unmanipulated haploidentical bone marrow transplantation and posttransplantation cyclophosphamide for hematologic malignancies after myeloablative conditioning. *Biol Blood Marrow Transplant.* (2013) 19:117–22. doi: 10.1016/j.bbmt.2012.08.014
13. Cieri N, Greco R, Crucitti L, Morelli M, Giglio F, Levati G, et al. Post-transplantation cyclophosphamide and sirolimus after haploidentical hematopoietic stem cell transplantation using a treosulfan-based myeloablative conditioning and peripheral blood stem cells. *Biol Blood Marrow Transplant*. (2015) 21:1506–14. doi: 10.1016/j.bbmt.2015.04.025
14. Solomon SR, Solh M, Morris LE, Holland HK, Bashey A. Myeloablative conditioning with PBSC grafts for T cell-replete haploidentical donor transplantation using posttransplant cyclophosphamide. *Adv Hematol.* (2016) 2016:9736564. doi: 10.1155/2016/9736564
15. Brunstein CG, Fuchs EJ, Carter SL, Karanes C, Costa LJ, Wu J, et al. Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. *Blood*. (2011) 118:282–8. doi: 10.1182/blood-2011-03-344853
16. Reisner Y, Kapoor N, Kirkpatrick D, Pollack MS, Dupont B, Good RA, et al. Transplantation for acute leukaemia with HLA-A and B nonidentical parental marrow cells fractionated with soybean agglutinin and sheep red blood cells. *Lancet*. (1981) 2:327–31. doi: 10.1016/S0140-6736(81)90647-4
17. Reisner Y, Kapoor N, Kirkpatrick D, Pollack MS, Cunningham-Rundles S, Dupont B, et al. Transplantation for severe combined immunodeficiency with HLA-A, B, D, DR incompatible parental marrow cells fractionated by soybean agglutinin and sheep red blood cells. *Blood*. (1983) 61: 341–8. doi: 10.1182/blood.V61.2.341.bloodjournal612341
18. O'Reilly RJ, Brochstein J, Collins N, Keever C, Kapoor N, Kirkpatrick D, et al. Evaluation of HLA-haplotype disparate parental marrow grafts depleted of T lymphocytes by differential agglutination with a soybean lectin and E-rosette depletion for the treatment of severe combined immunodeficiency. *Vox Sang*. (1986) 51(Suppl 2):81–6. doi: 10.1159/000461564
19. Friedrich W, Hönig M. HLA-haploidentical donor transplantation in severe combined immunodeficiency. *Hematol Oncol Clin North Am*. (2011) 25:31–44. doi: 10.1016/j.hoc.2010.11.003
20. Martin PJ, Hansen JA, Torok-Storb B, Durnam D, Przepiorka D, O'Quigley J, et al. Graft failure in patients receiving T-cell-depleted HLA-identical allogeneic marrow transplants. *Bone Marrow Transplant*. (1988) 3:445–56.
21. Martelli MF, Aversa F, Bachar-Lustig E, Velardi A, Reich-Zelicher S, Tabilio A, et al. Transplants across human leukocyte antigen barriers. *Semin Hematol*. (2002) 39:48–56. doi: 10.1053/shem.2002.29255
22. Reisner Y, Ben-Bassat I, Douer D, Kaploon A, Schwartz E, Ramot B. Demonstration of clonable alloreactive host T cells in a primate model for bone marrow transplantation. *Proc Natl Acad Sci USA*. (1986) 83:4012–5. doi: 10.1073/pnas.83.11.4012
23. Kernan NA, Flomenberg N, Dupont B, O'Reilly RJ. Graft rejection in recipients of T-cell-depleted HLA-nonidentical marrow transplants for leukemia. Identification of host derived antidonor allocytotoxic T lymphocytes. *Transplantation*. (1987) 43:842–7. doi: 10.1097/00007890-198706000-00014
24. Uharek L, Gassmann W, Glass B, Steinmann J, Loeffler H, Mueller-Ruchholtz W. Influence of cell dose and graft-versus-host reactivity on rejection rates after allogeneic bone marrow transplantation. *Blood*. (1992) 79:1612–21. doi: 10.1182/blood.V79.6.1612.bloodjournal7961612
25. Bachar-Lusting E, Rachamim N, Li HW, Lan F, Reisner Y. Megadose of T cell-depleted bone marrow overcomes MHC barriers in sublethally irradiated mice. *Nat Med*. (1995) 1:1268–73. doi: 10.1038/nm1295-1268
26. Rachamin N, Gan J, Segall H, Krauthgamer R, Marcus H, Berrebi A, et al. Tolerance induction by "megadose" hematopoietic transplants: donor-type human CD34 stem cells induce potent specific reduction of host anti-donor cytotoxic T lymphocyte precursors in mixed lymphocyte culture. *Transplantation*. (1998) 65:1386–93. doi: 10.1097/00007890-199805270-00017
27. Gur H, Krauthgamer R, Berrebi A, Klein T, Nagler A, Tabilio A, et al. Tolerance induction by megadose hematopoietic progenitor cells: expansion of veto cells by short-term culture of purified human CD34(+) cells. *Blood*. (2002) 99:4174–81. doi: 10.1182/blood.V99.11.4174
28. Gur H, Krauthgamer R, Bachar-Lustig E, Katchman H, Arbel-Goren R, Berrebi A, et al. Immune regulatory activity of CD34+ progenitor cells: evidence for a deletion-based mechanism mediated by TNF-alpha. *Blood*. (2005) 105:2585–93. doi: 10.1182/blood-2002-11-3463
29. Aversa F, Tabilio A, Terenzi A, Velardi A, Falzetti F, Giannoni C, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. *Blood*. (1994) 84:3948–55.
30. Aversa F, Tabilio A, Velardi A, Cunningham I, Terenzi A, Falzetti F, et al. Treatment of high- risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med*. (1998) 339:1186–93. doi: 10.1056/NEJM199810223391702
31. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, et al. Full haplotype mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol*. (2005) 23:3447–54. doi: 10.1200/JCO.2005.09.117
32. Terenzi A, Aristei C, Aversa F, Perruccio K, Chionne F, Raymondi C, et al. Efficacy of fludarabine as an immunosuppressor for bone marrow transplantation conditioning: preliminary results. *Transplant Proc*. (1996) 28:3101.
33. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. (2002) 295:2097–100. doi: 10.1126/science.1068440
34. Ruggeri L, Aversa F, Martelli MF, Velardi A. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. *Immunol Rev*. (2006) 214:202–18. doi: 10.1111/j.1600-065X.2006.00455.x
35. Velardi A, Ruggeri L, Mancusi A, Aversa F, Christiansen FT. Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a

tool for immunotherapy of leukemia. *Curr Opin Immunol*. (2009) 21:525–30. doi: 10.1016/j.coi.2009.07.015
36. Moretta A, Pende D, Locatelli F, Moretta L. Activating and inhibitory killer immunoglobulin-like receptors (KIR) in haploidentical haemopoietic stem cell transplantation to cure high-risk leukaemias. *Clin Exp Immunol*. (2009) 157:325–31. doi: 10.1111/j.1365-2249.2009.03983.x
37. Ciccone E, Pende D, Viale O, Di Donato C, Tripodi G, Orengo AM, et al. Evidence of a natural killer (NK) cell repertoire for (allo) antigen recognition: definition of five distinct NK-determined allospecificities in humans. *J Exp Med*. (1992) 175:709–18. doi: 10.1084/jem.175.3.709
38. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood*. (2007) 110:433–40. doi: 10.1182/blood-2006-07-038687
39. Moretta L, Locatelli F, Pende D, Marcenaro E, Mingari MC, Moretta A. Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *Blood*. (2011) 117:764–71. doi: 10.1182/blood-2010-08-264085
40. Pende D, Marcenaro S, Falco M, Martini S, Bernardo ME, Montagna D, et al. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood*. (2009) 113:3119–29. doi: 10.1182/blood-2008-06-164103
41. Oevermann L, Michaelis SU, Mezger M, Lang P, Toporski J, Bertaina A, et al. KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. *Blood*. (2014) 124:2744–7. doi: 10.1182/blood-2014-03-565069
42. Stern M, Ruggeri L, Mancusi A, Bernardo ME, de Angelis C, Bucher C, et al. Survival after T cell-depleted haploidentical stem cell transplantation is improved using the mother as donor. *Blood*. (2008) 112:2990–5. doi: 10.1182/blood-2008-01-135285
43. Mancusi A, Ruggeri L, Urbani E, Pierini A, Massei MS, Carotti A, et al. Haploidentical hematopoietic transplantation from KIR ligand-mismatched donors with activating KIRs reduces nonrelapse mortality. *Blood*. (2015) 125:3173–82. doi: 10.1182/blood-2014-09-599993
44. Ciceri F, Labopin M, Aversa F, Rowe JM, Bunjes D, Lewalle P, et al. Acute Leukemia Working Party (ALWP) of European Blood and Marrow Transplant (EBMT) Group. A survey of fully haploidentical hematopoietic stem cell transplantation in adults with high-risk acute leukemia: a risk factor analysis of outcomes for patients in remission at transplantation. *Blood*. (2008) 112:3574–81. doi: 10.1182/blood-2008-02-140095
45. Sairafi D, Mattsson J, Uhlin M, Uzunel M. Thymic function after allogeneic stem cell transplantation is dependent on graft source and predictive of long term survival. *Clin Immunol*. (2012) 142:343–50. doi: 10.1016/j.clim.2011.12.001
46. Waller EK, Langston AA, Lonial S, Cherry J, Somani J, Allen AJ, et al. Pharmacokinetics and pharmacodynamics of anti-thymocyte globulin in recipients of partially HLA-matched blood hematopoietic progenitor cell transplantation. *Biol Blood Marrow Transplant*. (2003) 9:460–71. doi: 10.1016/S1083-8791(03)00127-7
47. Marek A, Stern M, Chalandon Y, Ansari M, Ozsahin H, Güngör T, et al. The impact of T-cell depletion techniques on the outcome after haploidentical hematopoietic SCT. *Bone Marrow Transplant*. (2014) 49:55–61. doi: 10.1038/bmt.2013.132
48. Schmidt-Hieber M, Schwarck S, Stroux A, Ganepola S, Reinke P, Thiel E, et al. Immune reconstitution and cytomegalovirus infection after allogeneic stem cell transplantation: the important impact of *in vivo* T cell depletion. *Int J Hematol*. (2010) 91:877–85. doi: 10.1007/s12185-010-0597-6
49. Perruccio K, Tosti A, Burchielli E, Topini F, Ruggeri L, Carotti A, et al. Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. *Blood*. (2005) 106:4397–406. doi: 10.1182/blood-2005-05-1775
50. Comoli P, Basso S, Labirio M, Baldanti F, Maccario R, Locatelli F. T cell therapy of Epstein-Barr virus and adenovirus infections after hemopoietic stem cell transplant. *Blood Cells Mol Dis*. (2008) 40:68–70. doi: 10.1016/j.bcmd.2007.06.020
51. Comoli P, Schilham MW, Basso S, van Vreeswijk T, Bernardo ME, Maccario R, et al. T-cell lines specific for peptides of adenovirus hexon protein and devoid of alloreactivity against recipient cells can be obtained from HLA-haploidentical donors. *J Immunother*. (2008) 31:529–36. doi: 10.1097/CJI.0b013e31817b9c6b
52. Ciceri F, Bonini C, Gallo-Stampino C, Bordignon C. Modulation of GvHD by suicide-gene transduced donor T lymphocytes: clinical applications in mismatched transplantation. *Cytotherapy*. (2005) 7:144–9. doi: 10.1080/14653240510018136
53. Marktel S, Magnani Z, Ciceri F, Cazzaniga S, Riddell SR, Traversari C, et al. Immunologic potential of donor lymphocytes expressing a suicide gene for early immune reconstitution after hematopoietic T-cell-depleted stem cell transplantation. *Blood*. (2003) 101:1290–8. doi: 10.1182/blood-2002-08-2351
54. Ciceri F, Bonini C, Stanghellini MTL, Bondanza A, Traversari C, Salomoni M, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol*. (2009) 10:489–500.
55. Vago L, Oliveira G, Bondanza A, Noviello M, Soldati C, Ghio D, et al. T-cell suicide gene therapy prompts thymic renewal in adults after hematopoietic stem cell transplantation *Blood*. (2012) 120:1820–30. doi: 10.1182/blood-2012-01-405670
56. Zhou X, Dotti G, Krance RA, Martinez CA, Naik S, Kamble RT, et al. Inducible caspase-9 suicide gene controls adverse effects from alloreplete T cells after haploidentical stem cell transplantation. *Blood*. (2015) 125:4103–13. doi: 10.1182/blood-2015-02-628354
57. Zhang P, Tey SK. Adoptive T cell therapy following haploidentical hematopoietic stem cell transplantation. *Front Immunol*. (2019) 10:1854. doi: 10.3389/fimmu.2019.01854
58. Perruccio K, Topini F, Tosti A, Carotti A, Aloisi T, Aversa F, et al. Photodynamic purging of alloreactive T cells for adoptive immunotherapy after haploidentical stem cell transplantation. *Blood Cells Mol Dis*. (2008) 40:76–83. doi: 10.1016/j.bcmd.2007.06.022
59. Mielke S, Nunes R, Rezvani K, Fellowes VS, Venne A, Solomon SR, et al. A clinical-scale selective allodepletion approach for the treatment of HLA-mismatched and matched donor-recipient pairs using expanded T lymphocytes as antigen-presenting cells and a TH9402-based photodepletion technique. *Blood*. (2008) 111:4392–402. doi: 10.1182/blood-2007-08-104471
60. Perruccio K, Topini F, Tosti A, Carotti A, Burchielli E, Ruggeri L, et al. Optimizing a photoallodepletion protocol for adoptive immunotherapy after haploidentical SCT. *Bone Marrow Transplant*. (2012) 47:1196–200. doi: 10.1038/bmt.2011.237
61. Roy DC, Lachance S, Cohen S, Delisle JS, Kiss T, Sauvageau G, et al. Allodepleted T-cell immunotherapy after haploidentical haematopoietic stem cell transplantation without severe acute graft-versus-host disease (GVHD) in the absence of GVHD prophylaxis. *Br J Haematol*. (2019) 186:754–66. doi: 10.1111/bjh.15970
62. Di Ianni M, Del Papa B, Cecchini D, Bonifacio E, Moretti L, Zei T, et al. Immunomagnetic isolation of CD4+CD25+FoxP3+ natural T regulatory lymphocytes for clinical applications. *Clin Exp Immunol*. (2009) 156:246–53. doi: 10.1111/j.1365-2249.2009.03901.x
63. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*. (2011) 117:3921–8. doi: 10.1182/blood-2010-10-311894
64. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood*. (2014) 124:638–44. doi: 10.1182/blood-2014-03-564401
65. Bader P, Soerensen J, Jarisch A, Ponstingl E, Krenn T, Faber J, et al. Rapid immune recovery and low TRM in haploidentical stem cell transplantation in children and adolescence using CD3/CD19 depleted stem cells. *Best Pract Res Clin Haematol*. (2011) 24:331–7. doi: 10.1016/j.beha.2011.04.005
66. Federmann B, Bornhauser M, Meisner C, Kordelas L, Beelen DW, Stuhler G, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: a phase II study. *Haematologica*. (2012) 97:1523–31. doi: 10.3324/haematol.2011.059378

67. Chaleff S, Otto M, Barfield RC, Leimig T, Iyengar R, Martin J, et al. A large-scale method for the selective depletion of alphabeta T lymphocytes from PBSC for allogeneic transplantation. *Cytotherapy*. (2007) 9:746–54. doi: 10.1080/14653240701644000
68. Handgretinger R. New approaches to graft engineering for haploidentical bone marrow transplantation. *Semin Oncol*. (2012) 39:664–73. doi: 10.1053/j.seminoncol.2012.09.007
69. Handgretinger R. Negative depletion of CD3(+) and TcRalphabeta(+) T cells. *Curr Opin Hematol*. (2012) 19:434–9. doi: 10.1097/MOH.0b013e3283582340
70. Carding SR, Egan PJ. Gammadelta T cells: functional plasticity and heterogeneity. *Nat Rev Immunol*. (2002) 2:336–45. doi: 10.1038/nri797
71. Drobyski WR, Majewski D, Hanson G. Graft-facilitating doses of *ex vivo* activated gammadelta T cells do not cause lethal murine graft-versus-host disease. *Biol Blood Marrow Transplant*. (1999) 5:222–30. doi: 10.1053/bbmt.1999.v5.pm10465102
72. Lamb LS Jr, Lopez RD. gammadelta T cells: a new frontier for immunotherapy? *Biol Blood Marrow Transplant*. (2005) 11:161–8. doi: 10.1016/j.bbmt.2004.11.015
73. Locatelli F, Bauquet A, Palumbo G, Moretta F, Bertaina A. Negative depletion of α/β+ T cells and of CD19+ B lymphocytes: a novel frontier to optimize the effect of innate immunity in HLA-mismatched hematopoietic stem cell transplantation. *Immunol Lett*. (2013) 155:21–3. doi: 10.1016/j.imlet.2013.09.027
74. Airoldi I, Bertaina A, Prigione I, Zorzoli A, Pagliara D, Cocco C, et al. γδ T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-αβ+/CD19+ lymphocytes. *Blood*. (2015) 125:2349–58. doi: 10.1182/blood-2014-09-599423
75. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, et al. HLA-haploidentical stem cell transplantation after removal of alphabeta+ T and B cells in children with nonmalignant disorders. *Blood*. (2014) 124:822–6. doi: 10.1182/blood-2014-03-563817
76. Merli PBG, Algeri F, Sinibaldi M, Strocchio M, Li Pira L, Pagliara G, et al. Donor T cells genetically modified with a novel suicide gene (inducible caspase 9, iC9) expand and persist over time after post-allograft infusion in patients given αβ T-cell and B-cell depleted HLA-haploidentical allogeneic stem cell transplantation (αβ haplo-HSCT) contributing to accelerate immune recovery. *Blood*. (2017) 130:211. doi: 10.1182/blood.V130.Suppl_1.211.211
77. Al Malki MM, Horowitz M, Handgretinger R, Leung W, Roy DC, Huang XJ, et al. Proceedings from the Second Haploidentical Stem Cell Transplantation Symposium-Haplo2014, San Francisco, California, December 4, 2014. *Biol Blood Marrow Transplant*. (2016) 22:594–604. doi: 10.1016/j.bbmt.2016.01.001
78. Perko R, Kang G, Sunkara A, Leung W, Thomas PG, Dallas MH. Gamma delta T cell reconstitution is associated with fewer infections and improved event-free survival after hematopoietic stem cell transplantation for pediatric leukemia. *Biol Blood Marrow Transplant*. (2015) 21:130–6. doi: 10.1016/j.bbmt.2014.09.027
79. Ismail AS, Behrendt CL, Hooper LV. Reciprocal interactions between commensal bacteria and gammadelta intraepithelial lymphocytes during mucosal injury. *J Immunol*. (2009) 182:3047–54. doi: 10.4049/jimmunol.0802705
80. Shelikhova L, Ilushina M, Shekhovtsova Z, Shasheleva D, Khismatullina R, Kurnikova E, et al. αβ T cell-depleted haploidentical hematopoietic stem cell transplantation without antithymocyte globulin in children with chemorefractory acute myelogenous leukemia. *Biol Blood Marrow Transplant*. (2019) 25:e179–82. doi: 10.1016/j.bbmt.2019.01.023
81. Foell J, Schulte JH, Pfirstinger B, Troeger A, Wolff D, Edinger M, et al. Haploidentical CD3 or α/β T-cell depleted HSCT in advanced stage sickle cell disease. *Bone Marrow Transplant*. (2019) 54:1859–67. doi: 10.1038/s41409-019-0550-0
82. Prezioso L, Manfra I, Bonomini S, Schifano C, Segreto R, Monti A, et al. Haploidentical hematopoietic stem cell transplantation in adults using the αβTCR/CD19-based depletion of G-CSF-mobilized peripheral blood progenitor cells. *Bone Marrow Transplant*. (2019) 54(Suppl 2):698–702. doi: 10.1038/s41409-019-0608-z
83. Kaynar L, Demir K, Turak EE, Öztürk ÇP, Zararsiz G, Gönen ZB, et al. TcRαβ-depleted haploidentical transplantation results in adult acute leukemia patients. *Hematology*. (2017) 22:136–44. doi: 10.1080/10245332.2016.1238182
84. Sestili S, Labopin M, Ruggeri A, Velardi A, Ciceri F, Maertens J, et al. T-cell-depleted haploidentical stem cell transplantation results improve with time in adults with acute leukemia: a study from the Acute Leukemia Working Party of the European Society of Blood and Marrow Transplantation (EBMT). *Cancer*. (2018) 124:2142–50. doi: 10.1002/cncr.31310
85. Pierini A, Ruggeri L, Carotti A, Falzetti F, Piccinelli S, Saldi S, et al. The "ultimate" haploidentical transplantation for the elderly with high-risk acute myeloid leukemia. *Bone Marrow Transplant*. (2019) 54(Suppl 2):803–5. doi: 10.1038/s41409-019-0618-x
86. Solomon SR, St. Martin A, Shah NN, Fatobene G, Al Malki MM, Ballen KK, et al. Myeloablative vs reduced intensity T-cell-replete haploidentical transplantation for hematologic malignancy. *Blood Adv*. (2019) 3:2836–44. doi: 10.1182/bloodadvances.2019000627

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REVIEW
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Innate Immune Responses in the Outcome of Haploidentical Hematopoietic Stem Cell Transplantation to Cure Hematologic Malignancies

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In the context of allogeneic transplant platforms, human leukocyte antigen (HLA)-haploidentical hematopoietic stem cell transplantation (haplo-HSCT) represents one of the latest and most promising curative strategies for patients affected by high-risk hematologic malignancies. Indeed, this platform ensures a suitable stem cell source immediately available for virtually any patents in need. Moreover, the establishment in recipients of a state of immunologic tolerance toward grafted hematopoietic stem cells (HSCs) remarkably improves the clinical outcome of this transplant procedure in terms of overall and disease free survival. However, the HLA-mismatch between donors and recipients has not been yet fully exploited in order to optimize the Graft vs. Leukemia effect. Furthermore, the efficacy of haplo-HSCT is currently hampered by several life-threatening side effects including the onset of Graft vs. Host Disease (GvHD) and the occurrence of opportunistic viral infections. In this context, the quality and the kinetic of the immune cell reconstitution (IR) certainly play a major role and several experimental efforts have been greatly endorsed to better understand and accelerate the post-transplant recovery of a fully competent immune system in haplo-HSCT. In particular, the IR of innate immune system is receiving a growing interest, as it recovers much earlier than T and B cells and it is able to rapidly exert protective effects against both tumor relapses, GvHD and the onset of life-threatening opportunistic infections. Herein, we review our current knowledge in regard to the kinetic and clinical impact of Natural Killer (NK), γδ and Innate lymphoid cells (ILCs) IRs in both allogeneic and haplo-HSCT. The present paper also provides an overview of those new therapeutic strategies currently being implemented to boost the alloreactivity of the above-mentioned innate immune effectors in order to ameliorate the prognosis of patients affected by hematologic malignancies and undergone transplant procedures.

Keywords: innate lymphocytes, haploidentical hematopoietic stem cell transplantation, immune-reconstitution, natural killer cells, innate lymphoid cells, γδ T cells, alloreactivity

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INTRODUCTION

Allogeneic (allo-) hematopoietic stem cell transplantation (HSCT) represents the best curative approach for patients affected by high-risk hematologic malignancies and several genetic disorders (1). In the absence of human leukocyte antigen (HLA)-identical siblings, HLA-haploidentical (haplo) related donors are a source of hematopoietic stem cells (HSCs) immediately available for almost any patients in need (2).

However, the first developed protocols of haplo-HSCT were mainly associated with graft rejection, high degree of treatment-related mortality (TRM) and severe graft-vs.-host-disease (GvHD) due to the partial HLA-mismatch between donors and recipients. This poor clinical outcome was also worsened by the increased risk of developing opportunistic infections, a phenomenon associated with a delayed immune-reconstitution (IR) following the transplant. On the other hand, HLA-mismatch remarkably boosted the so-called Graft-vs.-Leukemia (GvL) effect that eradicates those malignant cells surviving conditioning regimes (3, 4). Hence, the mechanisms inducing both GvHD and GvL rely on immunologic alloreactivity that, indeed, represents the bad and good side of the same coin in both allogeneic and haplo-HSCT. The possibility to improve GvL while limiting life-threatening side effects have firmly driven the development of new clinical protocols of haplo-HSCT delivering better clinical outcomes. In this context, a better understanding of both kinetics and mechanisms of IR is key to improve the prognosis of patients undergone haplo-HSCT and limit its side effects (5–13).

Several lines of evidences clearly showed that a full recovery of adaptive immune responses in transplanted patients take long time. Indeed, adaptive B- and T-cell effector-functions are either lacking or not completely competent for several months after haplo-HSCT, thus leaving the patients in a deadly condition of immune-deficiency. On the other hand, innate immune cells reconstitute early after haplo-HSCT, thus ensuring a certain degree of immune-protections in the first days/weeks after the transplant (3, 14). In particular, neutrophils and monocytes recirculate at levels similar to those of healthy individuals 1 month after the infusion of HSCs, while innate lymphocyte IR starts from the 2nd week after the transplant (15–17). Nonetheless, quite a few cell compartments of innate immunity are greatly impaired in their functions early after haplo-HSCT (18, 19). This scenario enforced the implementation of graft manipulations in allo- and haplo-HSCT setting (i.e., αβ T and/or B cell depletion) able to preserve Natural Killer (NK), gamma-delta (γδ) T and innate lymphoid (ILCs), thus avoiding a prolonged immune suppression and speeding their IR early after the transplant (**Table 1**) (12, 26–28). In particular, NK and γδ T cells have been shown to recover faster in those recipients receiving αβ T cell depleted grafts rather than the conventional CD34^{pos} conventional counterparts in the context of the haplo-HSCT setting (25, 28, 29).

Graft vs. Host Diseases and Opportunistic Infections

One of the main complications affecting the positive clinical outcomes of allo-HSCT is still represented by the donor-derived alloreactive T cell responses against host tissues, a phenomenon inducing the onset of GvHD mainly affecting skin, gastrointestinal tract and liver (30, 31). Moreover, the different expression of tissue antigens between donors and recipients together with the clinical setting of induced immune-deficiency in recipients represent additional factors that remarkably worsen the impact of GvHD (32). In order to limit T cell alloreactivity, several haplo-HSCT platforms have been developed over the recent years (summarized in **Table 1**), including T-cell depleted (TCD) and T-cell replete (TCRep) approaches (5, 22, 25). Although the infusion of TCD grafts coupled with a mega-dose of CD34^{pos} peripheral blood HSCs (on average 10×10^6 cells/kg body weight) ensures high engraftment rates associated with potent GvL effect and reduced GvHD, the small number of residual T lymphocytes administered in recipients are still able to induce high degrees of TRM and to delay IR with a subsequent increased rates of opportunistic infection onsets (5, 33). Hence, alternative and more efficient TCRep approaches able to better target alloreactive T cells have been developed in haplo-HSCT setting. These new protocols employ the infusion of high doses of post-transplant cyclophosphamide (PT-Cy), an immune-suppressant drug that is able to deplete *in vivo* all alloreactive and proliferating T cells (34). This new PT-Cy TCRep strategy showed since from the beginning very good clinical outcomes in term of engraftment, decreased GvHD and a faster kinetic of IR. Indeed, while donor T cell infused at the time of the transplant mediates a strong GvL in the first days soon after the administration of HSCs, the removal of those alloreactive and proliferating donor-derived T cells clones by PT-Cy limited the onset of GvHD afterward. These TCRep protocols have been then further optimized by infusing colony-stimulation factor (G-CSF)-primed grafts, by depleting *in vivo* selective T cell populations and by using a combination of other immune-suppressive agents (24, 35, 36).

Both the induced clinical condition of immune-deficiency early after allo- and haplo- HSCT and the delayed/aberrant IR facilitate the occurrence of opportunistic infections that greatly affect the quality and duration of life. Human cytomegalovirus (HCMV) is one of the most aggressive opportunistic microbes in allogeneic transplant including haplo-HSCT. Indeed, while HCMV infection is often asymptomatic or associated with mild flu-like symptoms in immune-competent hosts, its reactivation or *de novo* infection occurs in more than 50% of patients undergone haplo-HSCT within the first 3 months after the procedure and it remains a major cause of morbidity and mortality especially in TCD procedures (22, 37–45). Although the efficacy of the novel antiviral therapies decreased the incidence of HCMV infections/reactivations (46), this still represents one of main complications of allo-HSCT (47). In this regard, a careful selection of donors is recommended particularly within the haplo-HSCT setting, since their mismatch with the HCMV-serostatus of recipients

TABLE 1 | Main results of different haplo-HSCT protocols with relative clinical outcomes and immunological recovery.

Sample size and disease	haplo-HSCT platform	Conditioning	Relapse/NRM	aGvHD/cGvHD	Clinical outcomes	Immune-reconstitution findings	References
67 AML 37 ALL	G-CSF and TCD using CD34+ cell immunoselection	TBI Thiotepa Fludarabine ATG	Engraftment: 99% Relapse: 13,6% NRM: 36.5%	aGvHD: 8% cGvHD: 7.1%	EFS rate for AML: 48% ± 8% EFS rate for ALL: 46% ± 10%	CD4+ T cell count: from 100 ± 40/mm^3 to 200 ± 20/mm^3 for 10 months; CD8+ T cell count: 230 ± 80/mm^3 day +60; 570 ± 80/mm^3 on day +300; CD16+ NK cell count: 400/mm^3 stably by day +30	(5)
66 ALL 51 AML 47 CML 7 MDS	NMAC TCRep	ATG CsA (d −9) MMF (d −9 to +30) Methotrexate (d +1, +3, +6, +11)	Probability of relapse: 12% at 2 years for standard-risk Probability of relapse: 39% at 2 years for high-risk	aGvHD (III–IV): 23% cGvHD: 47%	DFS: 68% at 2 years for standard-risk DFS: 42% at 2 years for high-risk	Neutrophil counts recover between 13 and 14 days; quick recovery of NK cells; CD8+ T-cell recovery starts at 2nd month after the transplant; B- cell reconstitution starts at 6th month; CD4+ T-cell recovery is slower and can require till 1 year	(20, 21)
67 hematologic malignancies 1 paroxysmal nocturnal hemoglobinuria	NMAC TCRep	Cy (d −6, −5, +3, or +3/+4), fludarabine (d −6 to −2) TBI (d −1) tacrolimus MMF	Probabilities of relapse:51% at 1 year NRM: 4% at days 100; 15% at 1 year Graft failure: 13%	aGVHD (II-IV): 34% at day 200 aGVHD (III-IV): 6% at day 200	OS: 46% at 1 year; 36% at 2 years EFS: 34% at 1 year; 26% at 2 years	The median times to neutrophil recovery (>500/μL): day +15; The median times to platelet recovery (>20,000/μL): day +24	(22)
52 AML 16 ALL 15 MDS	Unmanipulated G-CSF mobilized PB with *in vivo* TCD MAC or RIC		NRM: 14% (MAC) or 9% (RIC) at 3 years Incidence of relapse: 44% (MAC) or 58% (RIC) at 3 years	aGVHD (II-IV): 16% (MAC) or 19% (RIC) cGvHD: 30%(MAC) or 34% (RIC) at 3 years	OS: 45% (MAC) or 46% (RIC)	Platelet count: 20,000/ul at 17 days; NK count: >100/ul from 3 months; CD8 count: >200/ul from 3 months; CD4+ count: >200/ul from at 1 year	(23)
57 AML 14 ALL CML 1 MM 8 HL 4 MDS 2 MFI NHL 1 Plasma Cell Leukemia	G-CSF primed, unmanipulated BM MAC = 68 or RIC = 29	TBF ATG Methotrexate CsA MMF basiliximab	TRM: 36 ± 65% (MAC) or 28 ± 9% (RIC) relapse: 22 ± 6% (MAC) or 45 ± 11% (RIC)	100 day Cumulative Incidence of aGvHD (II-IV): 31 ± 5% Cumulative Incidence of overall cGvHD: 12 ± 4% at 2 years	OS: 48 ± 7% (MAC) or 29 ± 10% (RIC) DFS: 43 ± 7% (MAC) or 26 ± 10% (RIC)	100 day Cumulative Incidence of neutrophil engraftment: 94 ± 3% 100 day Cumulative Incidence of platelet engraftment: 84 ± 4%	(24)
80 acute leukemia (AL) in pediatric children	Negative depletion of αβ T and B cells MAC	ATG (d −3, −5)	2 graft failure Relapse: 24% NRM: 5%	aGVHD (I/II): 30% cGVHD-free survival: 71% at 5 years	DFS: 71,4% (ALL) or 67.5% (AML)	CD3+ T cells/μL: 231 (1–1,618); CD4+ T cells/μL: 19 (0–442) and CD8+ T cells/μL: 24 (0–910) γδ T cells/μL: 181 (1–1,335) CD3-CD56+ NK cells/μL: 236 (47–1,813) CD19+ B cells/μL: 0 (0–20)	Clinical trial: NCT01810120 (25)

aGvHD, acute Graft vs. host disease; ALL, Acute lymphoid Leukemia; AML, Acute myeloid Leukemia; ATG, Anti-thymocyte globulin; BM, Bone marrow; CsA, Cyclosporine A; cGvHD, chronic Graft vs. host disease; CML, Chronic myeloid Leukemia; Cy, Cyclophosphamide; d, days; DFS, Disease free survival; EFS, Event free survival; G-CSF, Growth colony stimulating factor; HL, Hodgkin lymphoma; MAC, Myeloablative conditioning; MDS, Myelodisplastic syndrome; MFI, Myelofibrosis; NHL, Non-Hodgkin lymphoma; MM, multiple myeloma; MMF, Mycophenolate mofetil; NMAC, Non-myeloablative conditioning; NMR, Non relapse mortality; OS, Overall survival; PB, Peripheral blood; RIC, Reduced intensity conditioning; TBI, Total body irradiation; TBF, Thiotepa, Busilvex, Fludarabine; TCD, T cell depletion; TCRep, T cell repletion. In immune-reconstitution findings column the cell counts are defined as mean ± SD (first row) or mean (range) at 1 month (last row).

greatly impacts the incidence and the virulence of HCMV reactivation (47). In particular, HCMV-seropositive recipients receiving a graft from HCMV-seronegative donors have the highest risks to develop HCMV reactivations. On the other hand, administering grafts from HCMV-seropositive donors increases the degree of OS in HCMV-seropositive patients receiving myeloablative conditioning (40). Hence, also the type of conditioning regimens plays a role in HCMV reactivations after allo-HSCT. The protective effect of HCMV-seropositive donors toward HCMV-seropositive recipient is also associated with the transfer of anti-HCMV specific T cell immunity (48). The frequency of primary infections in HCMV-seronegative recipients receiving a transplant from a HCMV-seronegative donor is very low since the reactivating viral strains generally origin from recipients, while their control is mediated by donor-derived alloreactive immune cells (45, 49, 50). However, a few other studies denied any significant impact of donor serostatus on HCMV reactivation in recipients undergone allo-HSCT (51, 52), thus leaving this important matter open for further discussion and clinical investigations. HCMV infections/reactivations also greatly affects the pattern of IR of both adaptive (53, 54) and innate immune cells (55, 56). Hence, it is conceivable that the kinetic of ILCs, NK and γδ T cell IR after haplo-HSCT as well as their effector-functions are somewhat influenced by HCMV infections/reactivations (55–58).

INNATE LYMPHOID CELLS

ILCs are a heterogeneous population of non-B and non-T lymphocytes that originate from common lymphoid progenitors. Since they lack adaptive antigen receptors, ILCs are able to rapidly produce and secrete pro-inflammatory and regulatory cytokines in response to local injuries, inflammation, infections or commensal microbiota perturbations (59–61). Similar to T cells, ILCs have been grouped into cytotoxic and helper lymphocytes and classified into three distinct sub-populations on the basis of their cytokines production and of the transcription factors involved in their development. These cell subsets are named ILC1, ILC2, and ILC3 and functionally mirror the $CD4^{pos}$ T helper (Th)1, Th2, and Th17 cells, respectively. More recently, also NK cells have been grouped within ILC family and resemble the functions of $CD8^{pos}$ cytotoxic T cells (59, 62–65).

ILC1 are mainly involved in interferon-γ (IFN-γ) production and represent potent effectors against bacterial and viral infections (61, 66–68). Despite sharing these functions with NK cells, ILC1 are currently considered a distinct subpopulation in terms of phenotype, function and development. Indeed, ILC1 are generally poorly cytotoxic and, unlike NK cells, are found at high frequency in tonsil and gut epithelium (i.e., intraepithelial ILC1) (69). Instead, ILC1 are rare in peripheral blood (PB) where they can be easily distinguished from NK cells due to their lack of CD56 and CD94 surface expression (63, 70, 71). ILC2 are also mostly tissue-resident lymphocytes and their effector-functions are triggered by interleukin (IL)-25 and IL-33 produced by epithelial cells or other immune cells in response to parasite infections or to allergen exposure. Following activation, ILC2 produce and secrete type 2 cytokines including IL-4, IL-5, IL-9, and IL-13 (62, 72–75). Moreover, ILC2 contribute to the resolution of inflammation by producing amphiregulin (AREG), a member of the epidermal growth factor that helps repairing damaged tissues (76). ILC3 are mainly resident in the gut lamina propria but have been also found in skin, lung and liver (77). Two different ILC3 subsets have been identified based on the expression of the Natural Cytotoxic Receptor (NCR) NKp44 in humans and NKp46 in mice. Both NCR^{pos}/ and NCR^{neg}/ILC3 subsets are able to produce IL-17, a cytokine crucial for fungal infection resistance. NCR^{pos}/ILC3 can also secrete IL-22, an important cytokine that regulates the homeostasis of gut epithelium, prevents the dissemination of commensal bacteria and limits inflammatory response (78). Another subset of lymphocytes grouped within ILC family is represented by the so-called lymphoid tissue-inducer (LTi) cells that are mainly involved in lymphoid organogenesis in fetal life. However, LTi-like cells are present also in adult life where they facilitate the generation of secondary lymphoid organs (79). LTi/LTi-like cells also produce IL-22 and initiate protective immune responses against extracellular bacteria. However, these latter lymphocytes have been grouped separately from ILC3 since they have a unique transcriptional profile and are generated from distinct progenitors (80). Moreover, LTi/LTi-like cells are endowed with specialized functions related to adaptive immunity as they are involved in T and B cell development (79).

Despite their differences in term of phenotype and functions, several lines of evidence indicates that the helper-ILCs (i.e., ILC1, ILC2, and ILC3) have high degrees of cell plasticity, as each one of these three subsets can give rise to other members of the same family if cultured with the proper cytokine stimulation (81). Moreover, recent findings indicate that, although ILC1, ILC2, and ILC3 are mainly tissue-resident, they might traffic through the different organs by recirculating in the bloodstream. Indeed, gut-resident ILC2 can migrate into the lung and other peripheral tissues in response to helminthes or upon IL-25 stimulation to either fight the parasite infections or to contribute to tissue repair (82). This experimental evidence suggests that helper-ILCs, other than exerting anti-microbial responses and tissue remodeling in those organs where they reside under homeostatic conditions, can also mediate a protective role against tissue damage in different anatomic compartments following exposure to inflammatory stimuli. In the context of allo-HSCT, this phenomenon is highly relevant in the mucositis induced by chemo/radiotherapies, in the development of GvHD and in response to infections. However, little is known about the role(s) played by ILCs in the pathogenesis of hematologic malignancies as well as in the clinical outcomes of transplantation. Indeed, very few studies have addressed the role of immune- reconstituting ILC in the context of allo-HSCT (83), while their functions in haplo-HSCT remain still completely unexplored. Thus, in the next paragraphs we will summarize the evidence on ILCs in allo-HSCT setting.

Immune-Reconstitution of Innate Lymphoid Cells

It has been recently disclosed that ILCs have a great clinical impact in patients affected by Acute Myeloid Leukemia (AML) either at disease onset or after chemo/radiotherapy and allo-HSCT (84, 85). In particular, there is a great reduction of circulating ILCs in AML, a phenomenon associated with a relative increase of ILC1 and a decrease of NCR^{pos}/ILC3. The overall frequencies of PB NCR^{pos}/ILC3 but not the ones of ILC1 are restored to normal levels in AML responders to chemotherapy. These quantitative changes of circulating ILCs in AML patients mirror their impaired abilities in producing IFN-γ and type 2 cytokines (85). Taken together, these data suggest that either leukemia burden or disease relapse markedly affect ILC development, a phenomenon also confirmed *in vitro* by co-culturing ILC precursors with AML blasts (86).

It has been also reported that conditioning regimens prior allo-HSCT deplete circulating ILCs that then undergo in recipients through a slow process of IR taking at last 6 months for a complete recover. In this setting, reconstituting ILCs show an increased expression of markers associated with tissue homing, such as the skin-homing receptors CLA and CCR10, the gut-homing molecules α4β7 and CCR6, the activation/tissue-residence marker CD69 and the cell proliferation nuclear protein Ki-67 (84). After 3 months from the transplant, the levels of circulating ILC2 are still strongly decreased compared to those of healthy subjects, while NCR^{pos}/ILC3 outnumber the other ILC subsets (84). These data suggest that ILC3 play a major role in ILC IR after allo-HSCT. In line with this working hypothesis, a study showed that the high amounts of IL-22 produced by ILC3 can enhance both thymic regeneration and a more rapid T cell IR in a $IL2^{-/-}$ mouse model receiving a TCD allo-HSCT (87).

It has been also reported that both conditioning regimens and different source of HSCs affects ILC IR after the transplant. This is of great importance in those children affected by severe combined immune deficiency (SCID) and carrying mutations of genes either encoding the common γ-chain subunit of IL-2 receptor or the tyrosine kinase JAK3. These patients lack all ILC subsets and experience an effective T cell IR following allo-HSCT only in the presence of myeloablative conditioning regimens (88). Instead, the administrations of cyclosporine or corticosteroids do not affect ILC IR (84). Another study showed in an *in vitro* setting that ILC3 IR is hampered by both pre- and post-transplant treatments with the mobilizing agent G-CSF (89). Moreover, it has been also reported that the generation of ILCs (especially NCR^{pos}/ILC3) is much higher when culturing *in vitro* HSCs from bone marrow (BM) and umbilical cord blood rather than their counterparts from PB following mobilization with G-CSF (89).

Innate Lymphoid Cells and Graft vs. Host Disease

Several lines of evidence demonstrated that ILCs play a key role in limiting the onset of GvHD after allo-HSCT. In particular, it has been shown in murine models that ILC3 have a great impact in protecting recipient gut epithelial cells from alloreactive responses exerted by donor immune cells. This phenomenon is mediated by the ILC3 high production of IL-22 (90). Indeed, IL-22 deficient mice undergone allo-HSCT suffer from severe intestinal GvHD and intestinal barrier disruption, while the administration of IL-22 in transplanted wild type animals limits the onset of intestinal GvHD and enhances both intestinal stem cell recovery and epithelial cell regeneration (91). In humans, increased frequencies of circulating NCR^{pos}/ILC3 early after allo-HSCT correlate with a lower incidence of intestinal GvHD. Notably, the ability to secrete high amounts of IL-22 by NCR^{pos}/ILC3 exerts a key role in the regeneration of the mucosal gut barrier after immune depletion following allo-HSCT, thus protecting from GvHD onset (92, 93). Moreover, higher expressions on recipients' circulating ILCs of both CD69 and α4β7 markers before the transplant reduce the risk of developing GvHD and can serve as good prognostic factors (84). Even increased frequencies of $CD69^{pos}$/ILC1 are associated with lower incidence of severe cutaneous GvHD since these cells express high levels of the skin homing markers CLA and CCR10. It has been also reported in murine models that type 2 cytokines play a protective role in GvHD development (92). Another reported mechanism protecting from GvHD is the ability of ILC2 to produce AREG that, in turn, boosts epithelial cell regeneration after the tissue damage induced by the conditioning regimens (76).

Innate Lymphoid Cells and Opportunistic Viral Infections

Although the role of ILCs in controlling infections in immune-competent individuals seem marginal, studies in immune-deficient mice showed that these innate lymphocytes can fight different pathogens (83, 94). However, very little if nothing is known in regard to their functional role in allo- and in haplo-HSCT setting. Since both T and B cell IR start to be effective and functional relevant only after a few months after haplo-HSCT, innate immune system certainly plays a key role in controlling opportunistic infections early after the transplant (19, 48, 95, 96). In this regard, while NK cells represent an immediate available source of IFN-γ in the bloodstream, ILC1 can provide large amounts of the same pro-inflammatory cytokine in tissues as reported in murine models of CMV, influenza, and Sendai infections (97, 98). Unlike ILC1, ILC2 are mainly involved in tissue damage repair during the resolution of the inflammatory process rather than in controlling the opportunistic infections (76, 99). Indeed, the proliferation and effector-functions of ILC2 are inhibited by both type I and II IFN that are largely produced during the course of viral infections (75, 100). Thus, high levels of IFN-γ produced by tissue-residence ILC1 not only control viral replication but also limit the dysregulation of ILC2 homeostasis.

NATURAL KILLER CELLS

NK cells are innate lymphocytes playing a major role in the immune-surveillance mainly against cancer and viral infections without a prior antigen sensitization and through the signal delivered by large families of inhibitory and activating NK cell receptors (aNKRs and iNKRs) (101).

iNKRs recognize, as their natural ligands, "self" HLA-I molecules expressed on the surface of all nucleated cells, ensuring both the recognition of autologous targets and a certain threshold

of immunologic tolerance especially at tissue levels. On contrary, tumor-transformed, viral infected, and heterologous cells lack or have reduced or express heterologous HLA-I molecules, respectively. NK cells can recognize these abnormalities on "non-self" and threatening targets due to the impaired or missing binding with iNKRs, whose downstream signaling is normally dominant over the activating stimuli driven by aNKRs in NK cells ("missing-self hypothesis"). The absence of this dominant inhibition shifts the balance toward NK cell activation via the engagement of aNKRs that binds their putative ligands on heterologous cell targets. These mechanisms trigger NK cell release of cytotoxic granules (i.e., perforin and granzymes) and secretion of anti-viral/pro-inflammatory cytokines for the clearance of both tumor and viral-infected cells (102–105).

The repertoire of NKRs is highly variable among different individuals and in different anatomic compartment and it is influenced by genetic factors, environmental exposure to non-self targets and tissue microenvironments (106, 107). Moreover, the phenotypic profiles of NK cells also depends by the so called "education/licensing" process that dictates the avidities of the interactions between iNKRs and their putative HLA ligands (108). The main classes of NKRs specific for HLA-I molecules include Killer Ig-like Receptors (KIRs) that recognize different HLA-A, -B, and -C allotypes (109) as well as the C-type lectin receptors CD94/NKG2A and CD94/NKG2C that bind the non-classical HLA-E molecules (110, 111). KIRs (known as CD158 molecules) represent a highly polymorphic family of NKRs that serve as regulators of development, tolerance and activation of NK cells (112). Interestingly, KIR superfamily includes both activating and inhibitory forms sharing homology in the extracellular domain, while differing for their cytoplasmatic tails. Activating KIRs (aKIRs) are characterized by a short intracellular domains that interact with adaptor signaling molecules carrying an Immunoreceptor Tyrosine-Based Activating Motif (ITAM) such as DAP-12 (113). On contrary, long cytoplasmatic tails containing Tyrosine-Based Inhibitory Motif (ITIM) distinguish inhibitory receptors (iKIRs) (109, 113, 114).

Similarly, the inhibitory C-type lectin receptor CD94/NKG2A is characterized by long intracellular tail containing ITIM motifs, while the trans-membrane domain of CD94/NKG2C interacts with the ITAM-containing adaptor molecule DAP-12 driving NK cell activation (115, 116). Among the other aNKRS driving the activation of NK cells there are the NCRs NKp30, NKp44, NKp46 together with the co-receptor NKp80 and 2B4 (117, 118).

CD16 (FCγRIII) is an immunoglobulin (Ig) receptor that, upon binding with the Fc portion of IgG antibodies, induces series of potent activating signals through the adaptor molecules CD3ζ and FcεRγ containing the activation ITAM motif. This down-stream pathway mediates the so-called antibody-dependent cell mediated cytotoxicity (ADCC) (119). The sequential expressions of CD16 together with KIRs, NCRs and C-type lectin receptors characterize the developmental stages, the effector-functions and the education of NK cells (120). The main steps of NK cell ontogenesis take place in BM niche starting from $CD34^{pos}$ HSCs but, differently from helper ILCs, these innate lymphocytes are mainly enriched in PB (121). Indeed, under homeostatic conditions, NK cells account up to 10% of total circulating lymphocytes and represent an heterogeneous population that can be subdivided into two main subsets according to the surface expression of CD56 and CD16 (122). $CD56^{bright}/CD16^{neg-low}$ ($CD56^{br}$) NK cells represent 5–15% of total circulating NK cells and are considered regulatory lymphocytes, as they produce high amounts of chemokines/cytokines and are involved in the cross-talk with other immune cells such as dendritic cells (DCs) and monocytes/macrophages (123–125). On the other hand, $CD56^{dim}/CD16^{pos}$ ($CD56^{dim}$) NK cells are the largest NK cell subset in PB (up to 95%) and mainly exert cytototoxic functions via the secretion of lytic granules (104, 126–128). $CD56^{br}$ and $CD56^{dim}$ are also considered two sequential stages of NK cell maturation with the latter subset being the terminally-differentiation one characterized by shortest telomere length (120, 121, 129, 130). $CD56^{br}$ NK cells usually show high levels of CD94/NKG2A, while almost lack KIRs (131). On contrary, $CD56^{dim}$ NK cells acquire KIR expression and loose CD94/NKG2A, thus being fully licensed end-stage effector cells (115, 132). Despite intense efforts in better disclosing human NK cell ontogenesis, the mechanisms tuning the appearance of NKRs and the different NK cell developmental stages remain to be elucidated (120).

NK Cell Immune-Reconstitution

Given the ability of NK cells to promptly mount effective alloreactive responses against tumor cells and pathogens, their kinetic and quality of IR certainly play important roles in determining the clinical outcome of allo- and haplo-HSCT. Indeed, delayed recoveries of these donor-derived alloreactive innate lymphocytes result in poor clinical outcomes of transplants (133, 134). As a matter of fact, NK cells are the first lymphocytes to appear soon after allo-and haplo-HSCTs and are essential for a better engraftment, to avoid tumor relapse and to limit the onsets of both GvHD and opportunistic viral infection. Moreover, the possibility to follow human NK cell IR in this unique *in vivo* setting is key in disclosing the several unknown mechanisms and patterns of their ontogenesis and differentiation (19, 135, 136). Regardless of the graft sources, NK cell chimerism in recipient is completely donor dependent after one month from haplo-HSCT. However, although the frequencies and absolute counts of circulating NK cells reach normal levels after few weeks post-transplant, their maturation and achievement of efficient effector-functions takes much longer (6, 15, 19, 130, 135, 137). Similar results have been observed also in recipients receiving HLA-matched HSCT, where reconstituting NK cells remain immature for more than 6 months after the infusion of HSCs. These phenomena are associated with functional defects that do not ensure an optimal protection against HCMV infections/reactivations, GvHD onset and tumor relapse in the first year after HSCT (138).

Reconstituting NK cells derive from $CD34^{pos}$ progenitors rather than from already mature NK cells infused with the graft. Indeed, the PT-Cy eliminates proliferating alloreactive NK cells in haplo-HSCT as they have an even higher proliferation rate compared to T cells in the first days after the graft infusion and before the Cy administration. The 2^{nd} wave of

proliferating donor-derived NK cells occurs after 15 days from haplo-HSCT and these new innate lymphocytes display an immature phenotype, thus confirming that they are *de novo* generated from donor HSCs (96). Indeed, $CD56^{br}$ NK cell subset appears much earlier that terminally differentiated $CD56^{dim}$, while the NK cell surface distribution of both CD56 and CD16 return similar to that of healthy donors only several months later (6, 19, 96, 120, 139–141). Unexpectedly though, we recently reported that the subset of reconstituting donor-derived NK cells expanded at the highest frequency in the first weeks after haplo-HSCT is characterized by an unconventional $CD56^{dim}/CD16^{neg-low}$ phenotype ($unCD56^{dim}$). This neglected NK cell population is present at very low frequency under homeostatic conditions, but plays a key role in the IR and in the clinical outcome of haplo-HSCT. In particular, although armed to be cytotoxic and carrying large amounts of perforin and granzymes, $unCD56^{dim}$ NK cells are highly defective in their killing activities due to the transient high expression of CD94/NKG2A receptor. Hence, this C-lectin type receptor functions as an inhibitory checkpoint that renders donor-derived $unCD56^{dim}$ NK cells anergic against residual tumor cells, recipients T cells and Antigen Presenting Cells (APC). This NK cell status early after haplo-HSCT makes recipients more at risk to undergo tumor relapse and to develop acute (a) GvHD. Similar transient high surface levels of CD94/NKG2A have been observed also on terminally-differentiated and cytotoxic $CD56^{dim}$ NK cells that start to reconstitute from the 2^{nd} month after the transplants and subsequent to the appearance of $unCD56^{dim}$ NK cells (19, 135, 139, 142). This gained knowledge paved the ground for a novel therapeutic approach targeting CD94/NKG2A in order to unleash NK cell cytotoxicity in haplo-HSCT.

NK Cells and Graft vs. Host Diseases

The HLA-mismatch between donor and recipient cells allow donor-derived and alloreactive NK cells to both limit the onset of GvHD and to prevent graft rejection in allo- and haplo-HSCT (143, 144). Indeed, several studies directly correlated an efficient NK cell IR in allogeneic transplant with the reduced incidence of relapse as well as with decreased rates of opportunistic infections in the presence of lower TRM and increased OS (134, 145, 146). In contrast, low frequencies of NK cells in the first weeks after allo-HSCT are associated with increased non-relapse mortality, shorter OS and higher degrees of opportunistic infections (133, 145). This clinical evidence underline the importance of NK cell IR in shaping the clinical outcomes of allogeneic transplants and its possible exploitation for developing novel therapeutic strategies (2, 135, 147). However, the exact NK cell-mediated mechanism preventing GvHD onset are not yet fully elucidated. One working hypothesis is that alloreactive NK cells could limit GvHD by killing donor T cells via the NKG2D-mediated recognition of stress-induced NKG2D-ligands on activated T lymphocytes (148, 149). Another study claimed that high frequency of NK cells in the first weeks after HSCT might prevent T cell proliferation through IL-10 production (150). Conversely, it has been also reported that NK-cell production of pro-inflammatory IFN-γ could promote tissue damage and consequent GvHD (151). Notably, also the quality of NK cells IR greatly affects the occurrence of GvHD after allo-HSCT. Indeed, higher surface levels of CD94/NKG2A on NK cells have been reported to limit aGvHD *in vivo* by inhibiting T cell proliferation and activation (152). Furthermore, increased frequencies of $CD94/NKG2C^{pos}$ NK cells are associated with a lower incidence of GvHD in allo-HSCT (153).

Even the NK cell maturation stage is important, as a recent report showed that those haplo-HSCT recipients developing GvHD display a more differentiated and activated NK cell phenotype (154). This evidence has been also further corroborated by other studies reporting that a reduction of circulating $CD56^{br}$ NK cells in the first 2 months after allo-HSCT is associated with higher incidence of aGvHD. This latter clinical correlation was so evident in the recruited cohorts of patients receiving allo-HSCT to be proposed as an early prognostic factor to predict GvHD (141, 143). Moreover, a higher ratio of T/NK during IR after phase correlates with a higher risk to develop both acute and chronic GvHD in haplo-HSCT (8).

Remarkably, the potential clinical benefits of reconstituting NK cells in haplo-HSCT might be influenced by pre- and post-conditioning treatments. In this regard, many studies performing adoptive transfer of NK cells after haplo-HSCT showed a reduced risk in aGvHD induction (151). Moreover, GvHD prophylaxis with Mycophenolate Mofetil has been demonstrated to inhibit NK cell proliferation and effector-functions (155, 156), thus affecting the NK cell mediated control of GvHD and opportunistic infections.

NK Cells and Viral Infections

The occurrence of an optimal quantitative and qualitative NK cell IR in haplo-HSCT is key for hampering the onset of life-threatening opportunistic infections. Indeed, lower frequencies of circulating donor-derived NK cells are associated with higher susceptibilities to develop viral infections, mainly HCMV (157). In turn, HCMV infections/reactivations are also able to influence NK cell homeostasis and differentiation by inducing the expansion of the $CD56^{neg}/CD16^{pos}$ ($CD56^{neg}$) NK cell subset (158, 159). While poorly represented in healthy individuals, $CD56^{neg}$ NK cells are present at high frequencies in active and chronic HIV-1 and HCV infections (58, 160, 161) and display impaired effector-functions due to their abnormal repertoire of NKRs (162, 163). Indeed, $CD56^{neg}$ NK cells are defective in the clearance of viral infections and express markers of cell exhaustion of their surface (164, 165). However, the ontogenesis and the impact of $CD56^{neg}$ NK cells in determining the clinical outcomes of allo- and haplo-HSCT are still being debated. Recent studies revealed that HCMV infections/reactivations are beneficial rather than detrimental on NK cell recovery upon haplo-HSCT. In particular, it has been reported that this virus can accelerate NK cell maturation and shape their NKR repertoire in haplo-HSCT by inducing the expansion of terminally-differentiated and alloreactive $CD56^{dim}$ NK cells which, in turn, exert potent GvL effects (166). Indeed, upon HCMV infections/reactivations,

$CD56^{dim}$ NK cells acquire a mature $NKG2C^{pos}/CD57^{pos}/NKG2A^{neg}/KIR^{pos}$ phenotype, thus becoming fully licensed to efficiently exert anti-viral and anti-tumor properties (i.e., production of IFN-γ and Tumor Necrosis Factor (TNF)-α) (167–169). On the contrary, NK cells from haplo-HSCT patients that do not experience HCMV infections/reactivations retain an immature phenotype characterized by high expressions of CD94/NKG2A (170).

These HCMV-induced $NKG2C^{pos}/CD57^{pos}/NKG2A^{neg}/KIR^{pos}/CD56^{dim}$ NK cell subset can persist even after 1 year from haplo-HSCT and show higher effector-functions when re-encountering the same antigen or following a proper activation with specific pro-inflammatory cytokines. These data suggest that HCMV infections/reactivations drive the expansion of NK cells with adaptive properties (167, 170–172). Similar features have been reported in murine models *in vivo*, where the murine CMV (MCMV) infection is responsible for the expansion of the so-called "memory-like" NK (ml-NK) cells that specifically recognize the viral glycoprotein m157 through the activating receptor Ly49H (173, 174). However, neither a univocal phenotype nor the receptor(s) able to specifically bind HCMV antigens have been clearly defined in human ml-NK cell and this is a matter currently being highly investigated in several models *in vitro* and *ex vivo*. In this regard, NKG2C has been proposed as the best putative candidate binding HCMV antigens, since those NK cells expressing this aNKR are the ones preferentially expanded following this viral infection (175, 176). In this regard, it has also been reported that the HCMV-encoded UL40 protein stabilizes HLA-E surface expression on target cells, thus favoring the recognition of viral-infected via the NKG2C/HLA-E interactions (159, 177). Moreover, another study claimed that proliferation/expansion of $NKG2C^{pos}$ NK cells requires additional signaling pathways including the one mediated by IL-12 produced by autologous monocyte (178). Despite all the above-mentioned experimental evidence, the primary role of NKG2C in the homeostasis and functional relevance of ml-NK cells is still unclear. Indeed, other subsets of $NKG2C^{neg}/KIR^{pos}$ NK cells are also expanded in response to HCMV infection and they are able as well to recognize viral-infected cells (106), thus suggesting the existence of additional aNKRs (i.e., KIRs) involved in the expansion of human ml-NK cells (179). Furthermore, NKG2C-deficient individuals can mount equivalent adaptive NK cell response against HCMV (180). In agreement, in patients receiving cord blood grafts from $NKG2C^{-/-}$ donors, HCMV infection is still able to promote NK cell maturation in the absence of this activating C-lectin type molecule. This latter experimental evidence further supports the current working hypothesis that other NKRs such aKIRs play a central role in the generation of ml-NK cells (181).

More recently, other studies demonstrated that the generation of ml-NK cells is associated with epigenetic reprogramming through a specific reconfiguration of adaptor molecules including tyrosine kinase SYK, the intracellular adaptor EAT-2, and the transmembrane adaptor protein FcεRγ. The gene expression of these three factors is regulated by the transcription factor promyelocytic leukemia zinc finger (PLZF), which is downregulated in the majority of ml-NK cells upon HCMV infections. As a matter of fact, the reduced expression of at least one of the above-mentioned signaling proteins is observed in the 50% of the HCMV-seropositive donors. Moreover, the reduced levels of PLZF also decreases the expression of IL-12 and IL-18 receptors, thus lowering NK cell responsiveness to these pro-inflammatory cytokines. The lack of FcεRγ, SYK, and EAT-2 in mature $CD56^{dim}$ NK cells is also correlated with the expansion of $NKG2C^{pos}$ NK cells upon HCMV infection (182–185). In this regard, $CD56^{neg}$ NK cells expanded in those patients receiving umbilical cord blood transplant and experiencing HCMV infection/reactivation are characterized by the downregulation of FcεRγ (186). In addition to the downregulation of PLZF, FcεRγ, SYK, and EAT-2, ml-NK cells share with cytotoxic $CD8^{pos}$ T cells similar genome-wide DNA methylation patterns (182), thus suggesting the existence of epigenetic determination programs associated with HCMV infections. Notably and similar to memory Th1 lymphocytes, the increased production of IFN-γ by ml-NK cells correlates with a stable demethylation of conserved non-coding sequence 1 of the *IFNG* locus (187).

Although there is a phenotypic heterogeneity of ml-NK cells following HCMV exposure, their rapid maturation in response to the viral challenges could favor not only the control of infection, but also NK cell alloreactivity against residual tumor cells (188). Hence, HCMV infection can represent a "natural" tool to generate ml-NK cells to then use for adoptive cellular immunotherapies (132, 189, 190). In this regard, newborn mice challenged with MCMV showed that ml-NK cells undergo expansion, release cytokines and provide a protective anti-tumor immune response in adoptive cell transfers (173). In humans, the expansion and the functional relevance of $NKG2C^{pos}$ ml-NK cells in HSCT recipients experiencing *de novo* viral infection or undergone HCMV reactivations also depends from donor serostatus. Indeed, the *in vivo* expanded $NKG2C^{pos}$ ml-NK showed higher cytokine productions in those recipients receiving grafts from HCMV-seropositive donors compared to their counterparts originated from grafts of HCMV-seronegative donors. However, $NKG2C^{pos}$ ml-NK cells also expand in the absence of detectable HCMV viremia when both donor and recipient are HCMV-seropositive. These data suggest that also human $NKG2C^{pos}$ ml-NK cells are transplantable and require exposure to either active or latent (subclinical) HCMV antigens in the recipients for the expansion of alloreactive NK cells from seropositive donors (191). Moreover, $NKG2C^{pos}$ ml-NK cells are able to produce high levels of IFN-γ following *in vitro* co-culture with K562 erytroleukemia cell line, thus supporting their high potential in GvL effect (192). Consistent with these findings, the adoptive transfer of donor-derived or cytokine-induced (i.e., activation with IL-12, IL-15, IL-18) ml-NK cells induces in the recipients affected by refractory AML the expansion of NK cells producing high levels of IFN-γ when encountering tumor cell targets (172).

Taken together these results suggest that ml-NK cells can be potentially exploited in order to both better control HCMV infection/reactivation and to enhance GvL (193).

γδ T CELLS

γδ T cells are a group of unconventional T cells that bridge the gap between innate and adaptive immunity. Similar to αβ T cells, γδ T cells develop in the thymus and express a somatically rearranged T cell receptor (TCR) consisting of a TCR-γ and a TCR-δ chains (65, 194–196). In humans, γδ T cells normally account for the 1–10% of circulating T lymphocytes, while in mucosal tissues and skin they constitute the major subset of resident T cells (194, 196). Different γδ T cell subsets can be identified based on the Vδ expression (Vδ1, Vδ2, Vδ3, and Vδ5) (195, 197). Under homeostatic conditions, 95% of circulating γδ T cells express Vδ2 TCR paired with Vγ9 chain, whereas in mucosa and skin γδ T cells mostly express Vδ1 or Vδ3 TCRs paired with various Vγ chains (195, 198–200).

γδ T cells are rapid responders to pathogens and tumor-transformed cells, since they do not require further peripheral maturation or extensive clonal expansion to initiate their effector-functions (194). Therefore, γδ T cells allow a prompt immune-surveillance in a MHC-independent manner through the recognition of a diverse array of antigens including peptides, sulfatides and phospholipids (194, 196, 199, 201, 202). Moreover, the γδ TCR can bind CD1d expressed by APC loaded with glycolipids and microbial lipids (203). In addition to their TCR, γδ T cells express an array of pattern-recognition receptors, such as toll-like receptors (TLRs) (201, 204), activating and inhibitory NKRs (201, 205, 206), the NCRs NKp30 and NKp44 (206, 207), the aNKR DNAM-1, the Fc receptor CD16 as well as the C-type lectin-like receptors NKG2D and CD94/NKG2A (195, 206, 208, 209). The presence of such receptor repertoire suggests a tight regulation of the TCR-mediated activity through an interplay between activating and inhibitory signaling downstream pathways (206).

Upon their activation, γδ T cells secrete high levels of Th1 cytokines (i.e., IFN-γ and TNF-α) modulating the responses of other neighboring immune effectors which, in turn, induce monocyte-derived DC maturation/activation and enhance antigen-specific αβ T cell responses (194, 195). Moreover, γδ T cells are able to directly lyse target cells by the release of granzymes and perforin and the engagement of FAS and TRAIL death receptors (195, 197, 210). As consequence of their high heterogeneity, γδ T cells are implied in diverse biological functions. First, these cells exert anti-tumor activities against various types of solid tumors and hematological malignancies (211). Since they represent the most abundant population among epithelial-resident lymphocytes in mucosal tissues and skin, γδ T cells are also the first line of defense against pathogens in these anatomic compartments (211, 212). Finally, several γδ T cell subtypes are involved in the induction of transplant immune-tolerance both in solid organ transplantation and in allo-HSCT (211, 213).

γδ T Cell Immune-Reconstitution

The growing interests on the role of γδ T cells IR in HSCT arose from their potential ability to perform GvL effects and fight opportunistic infections in the absence of GvHD (205, 211, 214). Indeed, pediatric and adult patients undergone haplo-HSCT and showing a long-term disease-free survival (DFS) were coupled with high frequencies of circulating γδ T cells (215, 216). γδ T cells are also the predominant T cell population reconstituting early after haplo-HSCT, with the Vδ2 cells showing a faster recovery compared with B and T lymphocytes in the PB of recipients receiving αβ and CD19 depleted grafts. In particular, it has been shown that the recovery of the complimentary determinant region 3 (CDR3) of the TCR δ chain is almost completed after 2 months from haplo-HSCT (25, 28, 29, 48, 95, 217). In the context of allo-HSCT, the majority of both donor-derived Vδ1 and Vδ2 cell subset recovering in the first weeks have a $CD27^{pos}/CD45RA^{neg}$ Central Memory (CM) phenotype and contribute to ensure an early protection against viruses, bacteria and residual tumor cells that survived the conditioning regimes (65). The current working hypothesis of a peripheral expansion of graft-derived mature γδ T cells is further supported by experimental evidence indicating that the same γδ T cell clones found in the donor are present in the recipient after the transplant (218). Later on, within a range of 14–60 days post-transplantation, the frequency of CM γδ T cells progressively decreases and it is counterbalanced by increase frequencies of naïve $CD27^{pos}/CD45RA^{pos}$ γδ T cells originated from donor infused HSCs (28, 65). This latter *de novo* generation of reconstituting γδ T cells is confirmed by the fact that, while the repertoire of the γ and δ chains is qualitatively comparable between donors and recipients, their clonotype is different (57).

γδ T cell IR after allo- and haplo-HSCT can be influenced by different variables including the conditioning regimen, the administration of immuno-suppressive agents, the GvHD prophylaxis and the onset of opportunistic infections (211). In this regard, it has been reported that stem cell mobilization with G-CSF in allo-HSCT induces higher frequencies of Vδ1 T cells endowed with potent alloreactivity against AML blasts (214). Moreover, also donor/recipient characteristics (i.e., gender, age, disease type, and graft source) affect γδ T cell IR too. Indeed, patients receiving a transplant from either matched related (MRD) or haplo-related donors have significant differences in the recovery of γδ T cells compared to matched unrelated donor (MUD) (215).

γδ T Cells and Graft vs. Host Diseases

It has been reported in allogenic HSCT that patients developing aGvHD show an increased frequency of reconstituting γδ T cells (219). However, this evidence has been denied by more recent findings indicating that absolute counts of γδ T cells do not influence the incidence and the severity of GvHD (65, 215). Instead, higher frequencies of donor-derived γδ T cells in the grafts seem to protect against the development of severe aGvHD (220). Similarly, patients receiving a TCD haplo-HSCT and showing increased frequencies of γδ T cells undergo longer DFS and OS compared to those with normal/decreased immune-reconstituting γδ T cells. These data corroborate the current consensus stating that γδ T cells can facilitate GvL effect without inducing GvHD (196, 216, 221, 222).

γδ T Cells in Viral Infections

The occurrence of high frequencies of reconstituting γδ T cells early after haplo-HSCT also protect from bacterial infections and show a decreased incidence of both viral and fungal infections (215). Indeed, pediatric patients, receiving αβ TCD grafts in haplo-HSCT setting have both reduced numbers of γδ T cells at day 30 post-transplant and higher incidence of HCMV infections/reactivations (65). At the same time, opportunistic infections can also shape the homeostasis and maturation of these cells (28, 55, 57, 195, 223). Indeed, patients undergoing allo-HSCT and experiencing HCMV reactivations display a preferential proliferation of specific Vδ1 and Vδ3 T cell clones, thus suggesting that γδ T cells are capable of adaptive responses through an oligoclonal selection of specific TCR repertoires (57). In particular, HCMV reactivation in haplo-HSCT patients has been associated with a specific expansion of terminally differentiated cytotoxic Vδ1 T expressing the effector memory $CD45RA^{pos}/CD27^{neg}$ (TEMRA) phenotype (28). This HCMV-induced expansion of TEMRA γδ T cells also enhance their anti-tumor functions both against hematological (28, 223) and solid (224) tumor cell targets *in vitro*. Taken together, these results suggest that the adoptive transfer of HCMV-specific Vδ1-donor γδ T cells can be used as a possible alternative to the common infusion of HCMV-specific αβ T cells (225). Indeed, this novel approach could promote viral immunity, protect from HCMV-related complications while contribute to prevent from leukemic relapses (214).

NOVEL THERAPEUTIC STRATEGIES TO IMPROVE IR UPON HSCT

The early protection and the limited side effects following HSCT render innate immune system a particularly attractive tool for adoptive cell therapy strategies. In this context, several approaches have been recently developed to improve NK and γδ T cell IR and to enhance their reactivity against cancer. These new therapeutic strategies include the targeting of checkpoint inhibitors, the stimulation with activating cytokines and genetic engineering of immune cells (**Table 2**) (**Figure 1**).

CHECKPOINT INHIBITOR

NK cells and γδ T lymphocytes share several receptors including NCRs and iNKRs as CD94/NKG2A (195, 206, 209). The use of monoclonal antibodies (mAbs) against inhibitory immune checkpoints represents a promising therapeutic approach for both hematologic and solid tumors (228, 229). Of particular relevance, the blockade of NKG2A binding to HLA-E has been demonstrated to unleash the effector-functions of both T and NK cells in different kind of tumors (230–233). These encouraging results have driven the development of humanized IgG4 anti-NKG2A mAb (IPH2201, monalizumab), currently under investigation in many clinical trials for the treatment of solid tumors (clinicaltrials.gov) (**Table 2**). Conversely, only one phase I clinical study is now investigating the potential role of IPH2201 in hematologic malignancies after HLA-identical transplantation (NCT02921685). In this regard, our recent data demonstrate that there is a clear clinic indication to extend the IPH2201 administration early after haplo-HSCT, thus targeting those hypo-functional NK cells expressing high levels of NKG2A with the aim of enhancing their alloreactivity (19). Moreover, given the fast recovery of γδ T lymphocytes following haplo-HSCT, the post-transplant infusion of IPH2201 could also positively impact their anti-tumor responses in synergy with NK cells before the acquisition of a full functional competence of the adaptive immune response (i.e., T and B cells).

Among other receptors regulating NK cell missing-self responses, KIRs cover an important place. Indeed, their clinical impact have been firstly shown in AML patients undergoing haplo-HSCT where the mismatch between KIRs and their ligands in the recipient has been exploited to promote alloreactive NK cell-mediated GvL effect (135). In this context, therapeutic anti-KIR mAb (IPH2101, 1-7F9, lirilumab) has been generated and its administration showed positive outcomes in AML and multiple myeloma (MM) patients (**Table 2**) (226, 227).

CYTOKINES

As anticipated, NK cell anti-tumor responses are finely governed by an array of NKRs tuning their balance between inhibition and activation. This gained knowledge allowed to implement several protocols of *in vitro* NK cell manipulation that use cytokines to regulate the aNKR repertoire, thus boosting their killing ability against tumor targets (**Table 2**).

IL-2 and IL-15 represent the first molecules used to induce the proliferation and increase the cytotoxic potential of both T and NK cells for adoptive cell transfer therapies in different tumor settings (234). Later on, IL-21, another cytokine involved in NK cell maturation (235) also gained clinical relevance for the treatment of hematologic malignancies. Indeed, a recent phase I clinical trial using K562-based feeder cells expressing membrane-bound chimeras of IL-21 (mbIL21) was conducted in patients affected by AML/myelodysplastic syndrome and demonstrated that the infusion of *ex vivo*–expanded NK cells from BM haplo-donor could control tumor relapse without major toxicity (236). Other clinical trials exploiting the same technology are currently ongoing for AML in haplo-HSCT setting (**Table 2**) (NCT02809092, NCT01787474, NCT01904136). In order to optimize NK cell expansion and effector-functions, other experimental approaches also tested the combination of different cytokines. In particular, the stimulation with IL-15, IL-12, and IL-18 together drive the expansion of a particular subset of NK cells displaying adaptive traits similar to those of ml-NK cells re-challenged by HCMV (172). The adoptive transfer of these donor-derived and cytokine-induced ml-NK cells in patients affected by refractory AML is associated with higher levels of IFN-γ encountering and eliminating tumor cell targets (172). Two clinical trials are currently administering cytokine-induced ml-NK cells in AML patients undergone haplo-HSCT (**Table 2**) (NCT02782546, NCT01898793).

The combination of IL-2 and IL-15 either alone or in synergy with other stimulant agents have been extensively used also to

TABLE 2 | Clinical trials targeting NK/γδ T cells in HSCT to cure patients with hematologic malignancies.

Therapeutic approach	Study title	Study phase/ status	Hematologic disease investigated	Cell sources/ targets	Drug/CAR construct	NCT number
Blocking mAbs	Study of a humanized antibody initiated 2 months After an HLA matched allogenic stem cell transplantation	Phase I/ recruiting	Hematologic malignancies (AML, ALL, MDS, MM, CLL, CML, myeloproliferative neoplasm, HD, NHD)	T, NK	Anti-NKG2A mAb, IPH2201, monalizumab	02921685
Blocking mAbs	Combination study of IPH2201 with Ibrutinib in patients with relapsed, refractory, or previously untreated chronic lymphocytic leukemia	Phase I-II/ active, not recruiting	Relapsed and refractory CLL	T, NK	Anti-NKG2A mAb, IPH2201, monalizumab	02557516
Blocking mAbs	Study on the anti-tumor activity, safety, and pharmacology of IPH2101 in patients with smoldering multiple myeloma	Phase II/ completed with results (226, 227)	Smoldering MM	NK	Anti-KIR mAb, IPH2101, Lirilumab	01222286
Blocking mAbs	Evaluation of activity, safety and pharmacology of IPH2101 a human monoclonal antibody in patients with multiple myeloma	Phase II/ completed with results (226, 227)	MM	NK	Anti-KIR mAb, IPH2101, Lirilumab	00999830
Blocking mAbs	A safety and tolerability extension trial assessing repeated dosing of anti-KIR (1-7F9) human monoclonal antibody in patients with acute myeloid leukemia	Phase I/ completed	AML	NK	Anti-KIR mAb, IPH2101, Lirilumab	01256073
Cytokines and drug stimulation	Interleukin-21 (IL-21)- expanded natural killer cells for induction of acute myeloid leukemia	Phase I-II/ recruiting	AML	NK	IL-21	02809092
Cytokines and drug stimulation	Donor natural killer cells in treating patients with relapsed or refractory acute myeloid leukemia	Phase I-II/ recruiting	AML	NK	IL-21	01787474
Cytokines and drug stimulation	Natural killer cells before and after donor stem cell transplant in treating patients with acute myeloid leukemia, myelodysplastic syndrome, or chronic myelogenous leukemia	Phase I-II/ recruiting	AML, MDS, CML	NK	IL-21	01904136
Cytokines and drug stimulation	Cytokine induced memory-like NK cell adoptive therapy after haploidentical donor hematopoietic cell transplantation	Phase II/ recruiting	AML	NK	IL12-IL15-IL18	02782546
Cytokines and drug stimulation	Cytokine-induced memory-like NK cells in Patients With Acute Myeloid Leukemia (AML) or Myelodysplastic Syndrome (MDS)	Phase I–II/ recruiting	AML	NK	IL12-IL15-IL18	01898793
Cytokines and drug stimulation	Zoledronic acid in combination with interleukin-2 to expand Vγ9Vδ2 T cells after T-replete haplo-identical allotransplant	Phase I/ recruiting	Hematologic malignancies	γδ T	Zol+IL2	03862833
Cytokines and drug stimulation	Expanded/activated gamma delta T-cell infusion following hematopoietic stem cell transplantation and post-transplant cyclophosphamide	Phase I/ not recruiting	AML, CML, ALL, MDS	γδ T	CliniMACS-Prodigy technology	03533816
Genetic engineering	Genetically modified haploidentical natural killer cell infusions for B-lineage acute lymphoblastic leukemia	Phase I/ completed	ALL	CAR-NK	Anti-CD19-BB-zeta	00995137
Genetic engineering	Pilot study of redirected haploidentical natural killer cell infusions for B-lineage acute lymphoblastic leukemia	Phase I/ suspended	ALL	CAR-NK	Anti-CD19-BB-zeta	01974479
Genetic engineering	Umbilical and Cord Blood (CB) derived CAR-engineered NK cells for b lymphoid malignancies	Phase I–II/ recruiting	ALL, CLL, NHL	CAR-NK	Anti-CD19-CD28-zeta-2A-iCasp9-IL15-transduced CB NK cells	03056339

ALL, Acute lymphoid Leukemia; AML, Acute myeloid Leukemia; CB, Cord blood; CLL, Chronic lymphoid Leukemia; CML, Chronic myeloid Leukemia; HL, Hodgkin lymphoma; IL, interleukin; MDS, Myelodisplastic syndrome; NHL, Non-Hodgkin lymphoma; mAb, monoclonal antibody; MM, multiple myeloma; Zol, Zoledronic acid.

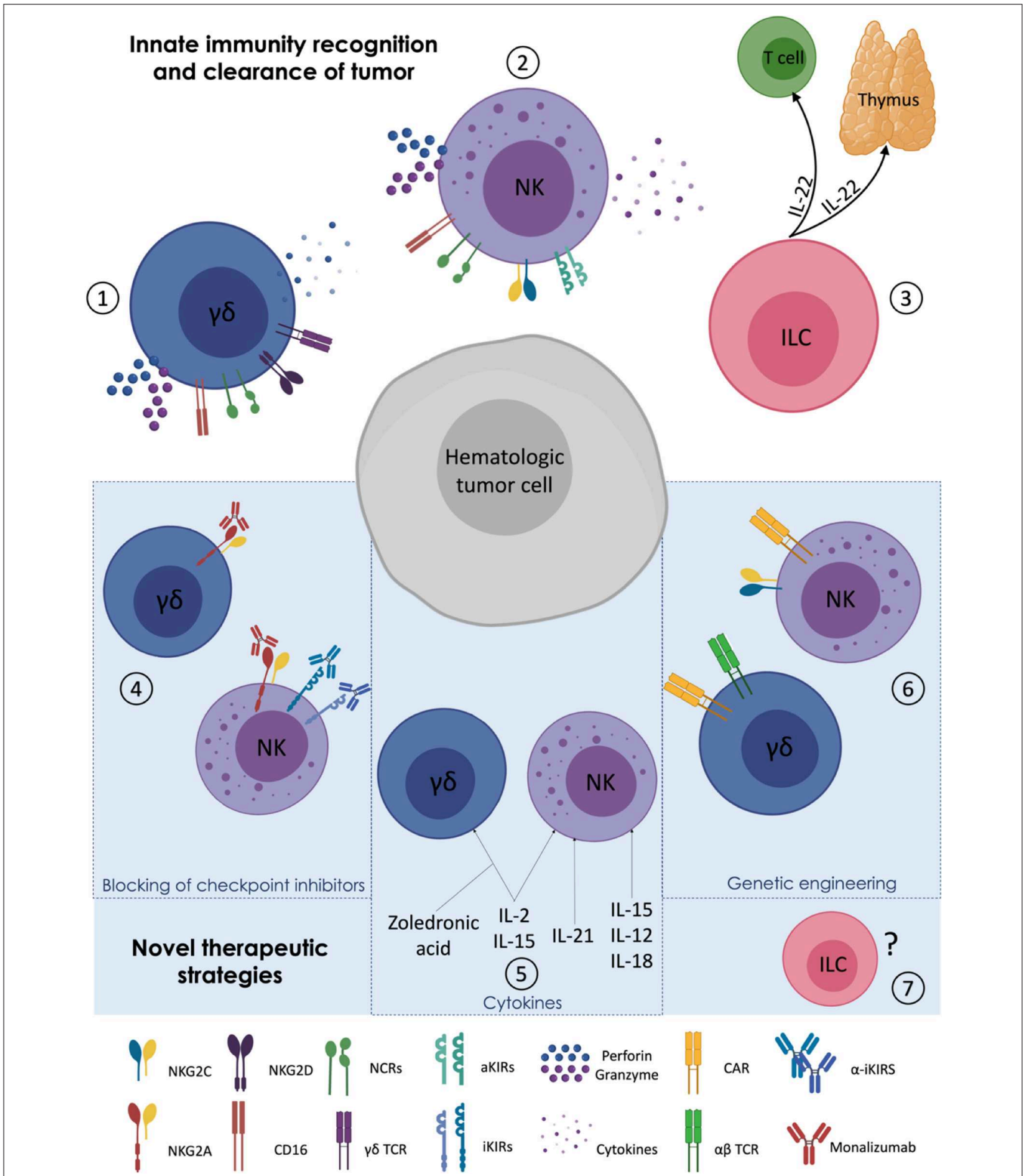

FIGURE 1 | Targeting γδ T, Natural Killer, and Innate Lymphoid cells in haplo-HSCT. (1–3) MHC-independent activation of innate immune cells: γδ T lymphocytes (1) and NK cells (2) can kill hematologic tumors by direct cytotoxicity and cytokine secretion. Innate Lymphoid cells (ILCs and ILC3 in particular) (3) play an indirect role in the clearance of tumors cells by improving both thymic regeneration and T cell maturation via their secretion of IL-22. (4–6) Novel therapeutic strategies implemented to enhance NK and γδ T cell alloreactivity against cancer: administration of monoclonal antibodies (mAbs) against NK cell inhibitory checkpoints (4); use of cytokines and zoledronic acid to activate γδ T cells (5); CAR editing and genetic engineering of γδ T and CAR on NK cells (6). (7) *Ad hoc* manipulation/editing/engineering of ILCs in transplant setting have not yet been explored.

expand γδ T cells (237). In this regard, one of the more promising protocols is represented by *in vivo* post-transplant administration of Zoledronic Acid (ZA) that improves the cytotoxicity of γδ T cells against leukemic cells (**Table 2**). This latter strategy relies on the use of ZA and IL-15 to expand terminally-differentiated and anti-tumor $CD45RO^{neg}/CD27^{neg}$ effector memory (TEMRA) Vδ2 cells. In this setting, the use of IL-15 is meant also to simultaneously boost the cytotoxicity and the proliferation of NK cells, thus targeting the two main anti-cancer effectors at the same time (28, 238, 239). In haplo-HSCT platforms, two very recent phase I studies propose to expand/activate γδ T cell prior (NCT03533816) or after (NCT03862833) cell infusion to provide innate GvL responses and to limit the onset of GvHD (**Table 2**).

GENETIC ENGINEERING

Genetic manipulation of immune cells allows the generation of highly specific anti-tumor effectors effectively targeting several tumor antigens. The introduction of chimeric antigen receptor (CAR)-T cells in HSCT opened new insight for the treatment of hematologic malignancies. Despite the very good clinical outcomes given by autologous CAR-T cell therapies against several tumors (240–243), the occurrence of life-threatening side effects such as tumor relapses (240, 244) and higher frequencies of GvHD and cytokine release syndrome onsets (245) have arisen major limitations in the use of allogeneic CAR-T cells. In this regard, engineering CAR-NK and CAR-γδ T cells may provide alternative procedures to improve their anti-tumor potentials, while overcoming allogeneic CAR-T cell therapy obstacles (**Table 2**) (139, 246–249). Notably, CAR-NK cells and CAR-γδ T cells retain the expression of their NKR repertoire and γδ TCR, respectively (214, 250). Hence, they can recognize tumor targets by their native receptors independently from CAR-restriction, thus reducing antigen-driven escape of tumor cells and further increasing their killing activities. CAR-NK cells are also characterized by relatively short life-span. If this latter feature certainly limits NK cell cytotoxicity over the time after transplantation, it can then prevent long-term side effects (such as cytopenia) that are observed upon CAR-T cell infusion (251).

A multitude of preclinical studies have tested the efficacy of CAR-NK cells against a variety of target antigens such as CD19 (252, 253) and CD20 (254, 255) for hematological malignancies as well as solid tumors. Another methodology used to promote the persistence of CAR-NK cell is to incorporate genes for IL-2 (256, 257) or IL-15 (258) within the CAR construct to constantly provide cytokine support to the CAR-transduced cells. In particular, this approach showed improved tumor control and prolonged survival in a mouse model of Raji lymphoma (258). These encouraging pre-clinical data opened new insights for the transfer of such protocols into human clinical trials such as the one that is optimizing the dose of IL-15-transduced CAR-NK cells for the treatment of B cell lymphoma (**Table 2**) (NCT03056339). Finally, genetic engineered CAR-NK cells mimicking ml-NK cells have been obtained redirecting NKG2C-mediated NK cell responses against cells expressing HLA-E. This protocol allows to overcome the dominant NKG2A-mediated inhibition, while boosting CAR-redirected NK cell activation via NKG2C (259).

Besides NK cells, also γδ T cells have been engineered against tumor targets using CAR technology (260). However, although CAR-γδ T cells were firstly introduced in 2004 (249), relatively few studies report their benefic potential in the treatment of hematologic and solid tumors.

Among these trials, PB-derived Vg9Vd2 T cells transduced with retroviral vectors encoding either disialoganglioside GD2- or CD19-specific CARs showed a higher capacity to secrete antigen-specific IFN-γ and to exert potent cytotoxicity against $GD2^{pos}$ neuroblastoma cells and $CD19^{pos}$ leukemic blasts *in vitro* (249). Furthermore, γδ T cells can be also transduced with exogenous αβ TCR directed against tumor associated antigens (214, 261). However, no clinical trials using CAR-γδ T cells have been initiated yet.

CONCLUDING REMARKS

Great efforts have been put in place to ameliorate the clinical outcome of allo-HSCT, to find an ideal donor for every patient in need and to limit the life-threatening complication of this transplant procedure. The development of haplo-HSCT platforms certainly represents a great step forward on these matters, although quite a few side effects, including the occurrence of GvHD and opportunistic infections, still affect the quality and the duration of life of these patients. In this regard, the quantity and quality of IR play a central role and require a deep understanding of all the mechanisms tuning the kinetic and the effector-functions of those immune cells that can better control the onset of tumor relapse, GvHD, and opportunistic infections. In this context, innate immune responses are key as they act immediately after the transplant. Several experimental and clinical studies clearly highlighted the importance to boost both adaptive and innate IR, ameliorate anti-tumor alloreactivity and develop alternative immunotherapy weapons against cancer.

The advances of current technologies have optimized the *ex vivo* expansion/activation of immune effectors and have selectively targeted checkpoint inhibitors also in the field of haplo-HSCT, where NK cells and γδ T lymphocytes early provide protection against cancers. Although helper ILCs could theoretically play a key role against tumors, the investigations of their clinical and functional impacts following HSCT are still in their infancy and must be deeper exploited. Our challenges and clinical perspectives over the next decade rely on our ability to give answers to the several important biological questions we still have on these matters.

AUTHOR CONTRIBUTIONS

EZ, MC, CD, and DM wrote the manuscript and approved the final version.

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REFERENCES

1. Esiashvili N, Pulsipher MA, Hematopoietic stem cell transplantation. In: Merchant TE, Kortmann RD, editors. *Pediatric Radiation Oncology*. Cham: Springer International Publishing (2018). p. 301–11.
2. Locatelli F, Pende D, Maccario R, Mingari MC, Moretta A, Moretta L. Haploidentical hemopoietic stem cell transplantation for the treatment of high-risk leukemias: how NK cells make the difference. *Clin Immunol.* (2009) 133:171–8. doi: 10.1016/j.clim.2009.04.009
3. Bosch M, Khan FM, Storek J. Immune reconstitution after hematopoietic cell transplantation. *Curr Opin Hematol.* (2012) 19:324–35. doi: 10.1097/MOH.0b013e328353bc7d
4. Mackall C, Fry T, Gress R, Peggs K, Storek J, Toubert AB, et al. Background to hematopoietic cell transplantation, including post transplant immune recovery. *Bone Marrow Transplant.* (2009) 44:457–62. doi: 10.1038/bmt.2009.255
5. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol.* (2005) 23:3447–54. doi: 10.1200/JCO.2005.09.117
6. Nguyen S, Dhedin N, Vernant JP, Kuentz M, Al Jijakli A, Rouas-Freiss N, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood.* (2005) 105:4135–42. doi: 10.1182/blood-2004-10-4113
7. Chen X, Hale GA, Barfield R, Benaim E, Leung WH, Knowles J, et al. Rapid immune reconstitution after a reduced-intensity conditioning regimen and a CD3-depleted haploidentical stem cell graft for paediatric refractory haematological malignancies. *Br J Haematol.* (2006) 135:524–32. doi: 10.1111/j.1365-2141.2006.06330.x
8. Chang YJ, Zhao XY, Huang XJ. Effects of the NK cell recovery on outcomes of unmanipulated haploidentical blood and marrow transplantation for patients with hematologic malignancies. *Biol Blood Marrow Transplant.* (2008) 14:323–34. doi: 10.1016/j.bbmt.2007.12.497
9. Chang YJ, Zhao XY, Huo MR, Xu LP, Liu DH, Liu KY, et al. Influence of lymphocyte recovery on outcome of haploidentical transplantation for hematologic malignancies. *Medicine.* (2009) 88:322–30. doi: 10.1097/MD.0b013e3181c167e2
10. Federmann B, Hagele M, Pfeiffer M, Wirths S, Schumm M, Faul C, et al. Immune reconstitution after haploidentical hematopoietic cell transplantation: impact of reduced intensity conditioning and CD3/CD19 depleted grafts. *Leukemia.* (2011) 25:121–9. doi: 10.1038/leu.2010.235
11. Ciurea SO, Mulanovich V, Jiang Y, Bassett R, Rondon G, McMannis J, et al. Lymphocyte recovery predicts outcomes in cord blood and T cell-depleted haploidentical stem cell transplantation. *Biol Blood Marrow Transplant.* (2011) 17:1169–75. doi: 10.1016/j.bbmt.2010.11.020
12. Oevermann L, Lang P, Feuchtinger T, Schumm M, Teltschik HM, Schlegel P, et al. Immune reconstitution and strategies for rebuilding the immune system after haploidentical stem cell transplantation. *Ann N Y Acad Sci.* (2012) 1266:161–70. doi: 10.1111/j.1749-6632.2012.06606.x
13. Park BG, Park CJ, Jang S, Chi HS, Kim DY, Lee JH, et al. Reconstitution of lymphocyte subpopulations after hematopoietic stem cell transplantation: comparison of hematologic malignancies and donor types in event-free patients. *Leuk Res.* (2015) 39:1334–41. doi: 10.1016/j.leukres.2015.09.010
14. Williams KM, Gress RE. Immune reconstitution and implications for immunotherapy following haematopoietic stem cell transplantation. *Best Pract Res Clin Haematol.* (2008) 21:579–96. doi: 10.1016/j.beha.2008.06.003
15. Chang YJ, Zhao XY, Huang XJ. Immune reconstitution after haploidentical hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2014) 20:440–9. doi: 10.1016/j.bbmt.2013.11.028
16. Chang YJ, Zhao XY, Huo MR, Xu LP, Liu DH, Liu KY, et al. Immune reconstitution following unmanipulated HLA-mismatched/haploidentical transplantation compared with HLA-identical sibling transplantation. *J Clin Immunol.* (2012) 32:268–80. doi: 10.1007/s10875-011-9630-7
17. Storek J, Dawson MA, Storer B, Stevens-Ayers T, Maloney DG, Marr KA, et al. Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation. *Blood.* (2001) 97:3380–9. doi: 10.1182/blood.V97.11.3380
18. Zimmerli W, Zarth A, Gratwohl A, Speck B. Neutrophil function and pyogenic infections in bone marrow transplant recipients. *Blood.* (1991) 77:393–9. doi: 10.1182/blood.V77.2.393.bloodjournal772393
19. Roberto A, Di Vito C, Zaghi E, Mazza MC, Capucetti A, Calvi M, et al. The early expansion of anergic NKG2A(pos)/CD56(dim)/CD16(neg) natural killer represents a therapeutic target in haploidentical hematopoietic stem cell transplantation. *Haematologica.* (2018) 103:1390–402. doi: 10.3324/haematol.2017.186619
20. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W, et al. Haploidentical hematopoietic stem cell transplantation without *in vitro* T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant.* (2006) 38:291–7. doi: 10.1038/sj.bmt.1705445
21. McCurdy SR, Luznik L. Immune reconstitution after T-cell replete HLA-haploidentical transplantation. *Semin Hematol.* (2019) 56:221–6. doi: 10.1053/j.seminhematol.2019.03.005
22. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
23. Lee KH, Lee JH, Lee JH, Kim DY, Seol M, Lee YS, et al. Reduced-intensity conditioning therapy with busulfan, fludarabine, and antithymocyte globulin for HLA-haploidentical hematopoietic cell transplantation in acute leukemia and myelodysplastic syndrome. *Blood.* (2011) 118:2609–17. doi: 10.1182/blood-2011-02-339838
24. Arcese W, Picardi A, Santarone S, De Angelis G, Cerretti R, Cudillo L, et al. Haploidentical, G-CSF-primed, unmanipulated bone marrow transplantation for patients with high-risk hematological malignancies: an update. *Bone Marrow Transplant.* (2015) 50(Suppl 2):S24–30. doi: 10.1038/bmt.2015.91
25. Locatelli F, Merli P, Pagliara D, Li Pira G, Falco M, Pende D, et al. Outcome of children with acute leukemia given HLA-haploidentical HSCT after αβ T-cell and B-cell depletion. *Blood.* (2017) 130:677–85. doi: 10.1182/blood-2017-04-779769
26. Bollard CM, Rooney CM, Heslop HE. T-cell therapy in the treatment of post-transplant lymphoproliferative disease. *Nat Rev Clin Oncol.* (2012) 9:510–9. doi: 10.1038/nrclinonc.2012.111
27. Maschan M, Shelikhova L, Ilushina M, Kurnikova E, Boyakova E, Balashov D, et al. TCR-alpha/beta and CD19 depletion and treosulfan-based conditioning regimen in unrelated and haploidentical transplantation in children with acute myeloid leukemia. *Bone Marrow Transplant.* (2016) 51:668–74. doi: 10.1038/bmt.2015.343
28. Airoldi I, Bertaina A, Prigione I, Zorzoli A, Pagliara D, Cocco C, et al. γδ T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR- αβ+/CD19+ lymphocytes. *Blood.* (2015) 125:2349–58. doi: 10.1182/blood-2014-09-599423

29. Lang P, Feuchtinger T, Teltschik HM, Schwinger W, Schlegel P, Pfeiffer M, et al. Improved immune recovery after transplantation of TCRαβ/CD19-depleted allografts from haploidentical donors in pediatric patients. *Bone Marrow Transplant*. (2015) 50(Suppl 2):S6–10. doi: 10.1038/bmt.2015.87
30. Vogelsang GB, Lee L, Bensen-Kennedy DM. Pathogenesis and treatment of graft-versus-host disease after bone marrow transplant. *Annu Rev Med*. (2003) 54:29–52. doi: 10.1146/annurev.med.54.101601.152339
31. Zeiser R, Blazar BR. Acute graft-versus-host disease - biologic process, prevention, and therapy. *N Engl J Med*. (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
32. Billingham RE, The biology of graft-versus-host reactions. *Harvey Lect*. (1966) 62:21–78.
33. Aversa F. T cell depleted haploidentical transplantation: positive selection. *Pediatr Rep*. (2011) 3(Suppl 2):e14. doi: 10.4081/pr.2011.s2.e14
34. Robinson TM, O'Donnell PV, Fuchs EJ, Luznik L. Haploidentical bone marrow and stem cell transplantation: experience with post-transplantation cyclophosphamide. *Semin Hematol*. (2016) 53:90–7. doi: 10.1053/j.seminhematol.2016.01.005
35. Mancusi A, Ruggeri L, Velardi A. Haploidentical hematopoietic transplantation for the cure of leukemia: from its biology to clinical translation. *Blood*. (2016) 128:2616–23. doi: 10.1182/blood-2016-07-730564
36. Lee CJ, Savani BN, Mohty M, Labopin M, Ruggeri A, Schmid C, et al. Haploidentical hematopoietic cell transplantation for adult acute myeloid leukemia: a position statement from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Haematologica*. (2017) 102:1810–22. doi: 10.3324/haematol.2017.176107
37. Dupont L, Reeves MB. Cytomegalovirus latency and reactivation: recent insights into an age old problem. *Rev Med Virol*. (2016) 26:75–89. doi: 10.1002/rmv.1862
38. Sinclair J, Sissons P. Latency and reactivation of human cytomegalovirus. *J Gen Virol*. (2006) 87:1763–79. doi: 10.1099/vir.0.81891-0
39. Landolfo S, Gariglio M, Gribaudo G, Lembo D. The human cytomegalovirus. *Pharmacol Ther*. (2003) 98:269–97. doi: 10.1016/S0163-7258(03)00034-2
40. Ljungman P, Brand R, Hoek J, de la Camara R, Cordonnier C, Einsele H, et al. Infectious diseases working party of the european group for, and T marrow, donor cytomegalovirus status influences the outcome of allogeneic stem cell transplant: a study by the European group for blood and marrow transplantation. *Clin Infect Dis*. (2014) 59:473–81. doi: 10.1093/cid/ciu364
41. Venton G, Crocchiolo R, Furst S, Granata A, Oudin C, Faucher C, et al. Risk factors of Ganciclovir-related neutropenia after allogeneic stem cell transplantation: a retrospective monocentre study on 547 patients. *Clin Microbiol Infect*. (2014) 20:160–6. doi: 10.1111/1469-0691.12222
42. Crocchiolo R, Bramanti S, Vai A, Sarina B, Mineri R, Casari E, et al. Infections after T-replete haploidentical transplantation and high-dose cyclophosphamide as graft-versus-host disease prophylaxis. *Transpl Infect Dis*. (2015) 17:242–9. doi: 10.1111/tid.12365
43. Ciurea SO, Mulanovich V, Saliba RM, Bayraktar UD, Jiang Y, Bassett R, et al. Improved early outcomes using a T cell replete graft compared with T cell depleted haploidentical hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2012) 18:1835–44. doi: 10.1016/j.bbmt.2012.07.003
44. Raiola AM, Dominietto A, Ghiso A, Di Grazia C, Lamparelli T, Gualandi F, et al. Unmanipulated haploidentical bone marrow transplantation and posttransplantation cyclophosphamide for hematologic malignancies after myeloablative conditioning. *Biol Blood Marrow Transplant*. (2013) 19:117–22. doi: 10.1016/j.bbmt.2012.08.014
45. Blyth E, Withers B, Clancy L, Gottlieb D. CMV-specific immune reconstitution following allogeneic stem cell transplantation. *Virulence*. (2016) 7:967–80. doi: 10.1080/21505594.2016.1221022
46. Marty FM, Ljungman P, Chemaly RF, Maertens J, Dadwal SS, Duarte RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. *N Engl J Med*. (2017) 377:2433–44. doi: 10.1056/NEJMoa1706640
47. Matthes-Martin S, Lion T, Aberle SW, Fritsch G, Lawitschka A, Bittner B, et al. Pre-emptive treatment of CMV DNAemia in paediatric stem cell transplantation: the impact of recipient and donor CMV serostatus on the incidence of CMV disease and CMV-related mortality. *Bone Marrow Transplant*. (2003) 31:803–8. doi: 10.1038/sj.bmt.1703927
48. Roberto A, Castagna L, Zanon V, Bramanti S, Crocchiolo R, McLaren JE, et al. Role of naive-derived T memory stem cells in T-cell reconstitution following allogeneic transplantation. *Blood*. (2015) 125:2855–64. doi: 10.1182/blood-2014-11-608406
49. Nichols WG, Corey L, Gooley T, Davis C, Boeckh M. High risk of death due to bacterial and fungal infection among cytomegalovirus. (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. *J Infect Dis*. (2002) 185:273–82. doi: 10.1086/338624
50. Gandhi MK, Wills MR, Okecha G, Day EK, Hicks R, Marcus RE, et al. Late diversification in the clonal composition of human cytomegalovirus-specific CD8+ T cells following allogeneic hemopoietic stem cell transplantation. *Blood*. (2003) 102:3427–38. doi: 10.1182/blood-2002-12-3689
51. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev*. (2009) 22:76–98. doi: 10.1128/CMR.00034-08
52. Crocchiolo R, Castagna L, Furst S, Devillier R, Sarina B, Bramanti S, et al. The patient's CMV serological status affects clinical outcome after T-cell replete haplo-HSCT and post-transplant cyclophosphamide. *Bone Marrow Transplant*. (2016) 51:1134–6. doi: 10.1038/bmt.2016.69
53. Itzykson R, Robin M, Moins-Teisserenc H, Delord M, Busson M, Xhaard A, et al. Cytomegalovirus shapes long-term immune reconstitution after allogeneic stem cell transplantation. *Haematologica*. (2015) 100:114–23. doi: 10.3324/haematol.2014.113415
54. Lugthart G, van Ostaijen-Ten Dam MM, Jol-van der Zijde CM, van Holten TC, Kester MG, Heemskerk MH, et al. Early cytomegalovirus reactivation leaves a specific and dynamic imprint on the reconstituting T cell compartment long-term after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2014) 20:655–61. doi: 10.1016/j.bbmt.2014.01.018
55. Knight A, Madrigal AJ, Grace S, Sivakumaran J, Kottaridis P, Mackinnon S, et al. The role of Vdelta2-negative γδ T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. *Blood*. (2010) 116:2164–72. doi: 10.1182/blood-2010-01-255166
56. Storek J. Immunological reconstitution after hematopoietic cell transplantation - its relation to the contents of the graft. *Exp Opin Biol Ther*. (2008) 8:583–97. doi: 10.1517/14712598.8.5.583
57. Ravens S, Schultze-Florey C, Raha S, Sandrock I, Drenker M, Oberdorfer L, et al. Human γδ T cells are quickly reconstituted after stem-cell transplantation and show adaptive clonal expansion in response to viral infection. *Nat Immunol*. (2017) 18:393–401. doi: 10.1038/ni.3686
58. Lugli E, Marcenaro E, Mavilio D. NK cell subset redistribution during the course of viral infections. *Front Immunol*. (2014) 5:390. doi: 10.3389/fimmu.2014.00390
59. Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity*. (2014) 41:354–65. doi: 10.1016/j.immuni.2014.09.005
60. Eberl G, Colonna M, Di Santo JP, McKenzie AN. Innate lymphoid cells. Innate lymphoid cells: a new paradigm in immunology. *Science*. (2015) 348:aaa6566. doi: 10.1126/science.aaa6566
61. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol*. (2016) 17:765–74. doi: 10.1038/ni.3489
62. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell*. (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
63. Trabanelli S, Gomez-Cadena A, Salome B, Michaud K, Mavilio D, Landis BN, et al. Human innate lymphoid cells. (ILCs): toward a uniform immune-phenotyping. *Cytomet Clin Cytom*. (2018) 94:392–9. doi: 10.1002/cyto.b.21614
64. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol*. (2013) 13:145–9. doi: 10.1038/nri3365
65. Park M, Im HJ, Lee YJ, Park N, Jang S, Kwon SW, et al. Reconstitution of T and NK cells after haploidentical hematopoietic cell transplantation using αβ T cell-depleted grafts and the clinical implication of γδ T cells. *Clin Transplant*. (2018) 32:e13147. doi: 10.1111/ctr.13147

66. Abt MC, Lewis BB, Caballero S, Xiong H, Carter RA, Susac B, et al. Innate immune defenses mediated by two ILC subsets are critical for protection against acute clostridium difficile infection. *Cell Host Microbe*. (2015) 18:27–37. doi: 10.1016/j.chom.2015.06.011
67. Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell*. (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
68. Shah SV, Manickam C, Ram DR, Reeves RK. Innate lymphoid cells in HIV/SIV infections. *Front Immunol*. (2017) 8:1818. doi: 10.3389/fimmu.2017.01818
69. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity*. (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
70. Roan F, Stoklasek TA, Whalen E, Molitor JA, Bluestone JA, Buckner JH, et al. CD4+ Group 1 innate lymphoid cells. (ILC) form a functionally distinct ILC subset that is increased in systemic sclerosis. *J Immunol*. (2016) 196:2051–62. doi: 10.4049/jimmunol.1501491
71. Lim AI, Di Santo JP. ILC-poiesis: ensuring tissue ILC differentiation at the right place and time. *Eur J Immunol*. (2019) 49:11–18. doi: 10.1002/eji.201747294
72. Koyasu S, Moro K, Tanabe M, Takeuchi T. Natural helper cells: a new player in the innate immune response against helminth infection. *Adv Immunol*. (2010) 108:21–44. doi: 10.1016/B978-0-12-380995-7.00002-1
73. Rauber S, Luber M, Weber S, Maul L, Soare A, Wohlfahrt T, et al. Resolution of inflammation by interleukin-9-producing type 2 innate lymphoid cells. *Nat Med*. (2017) 23:938–44. doi: 10.1038/nm.4373
74. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science*. (2015) 350:981–5. doi: 10.1126/science.aac9593
75. Moro K, Kabata H, Tanabe M, Koga S, Takeno N, Mochizuki M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol*. (2016) 17:76–86. doi: 10.1038/ni.3309
76. Monticelli LA, Osborne LC, Noti M, Tran SV, Zaiss DM, Artis D. IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin-EGFR interactions. *Proc Natl Acad Sci USA*. (2015) 112:10762–7. doi: 10.1073/pnas.1509070112
77. Montaldo E, Juelke K, Romagnani C. Group 3 innate lymphoid cells. (ILC3s): origin, differentiation, and plasticity in humans and mice. *Eur J Immunol*. (2015) 45:2171–82. doi: 10.1002/eji.201545598
78. Glatzer T, Killig M, Meisig J, Ommert I, Luetke-Eversloh M, Babic M, et al. RORgammat(+) innate lymphoid cells acquire a proinflammatory program upon engagement of the activating receptor NKp44. *Immunity*. (2013) 38:1223–35. doi: 10.1016/j.immuni.2013.05.013
79. Zhong C, Zheng M, Zhu J. Lymphoid tissue inducer-A divergent member of the ILC family. *Cytokine Growth Factor Rev*. (2018) 42:5–12. doi: 10.1016/j.cytogfr.2018.02.004
80. Ishizuka IE, Chea S, Gudjonson H, Constantinides MG, Dinner AR, Bendelac A, et al. Single-cell analysis defines the divergence between the innate lymphoid cell lineage and lymphoid tissue-inducer cell lineage. *Nat Immunol*. (2016) 17:269–76. doi: 10.1038/ni.3344
81. Colonna M. Innate lymphoid cells: diversity, plasticity, and unique functions in immunity. *Immunity*. (2018) 48:1104–17. doi: 10.1016/j.immuni.2018.05.013
82. Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science*. (2018) 359:114–9. doi: 10.1126/science.aam5809
83. Vacca P, Montaldo E, Croxatto D, Moretta F, Bertaina A, Vitale C, et al. NK cells and other innate lymphoid cells in hematopoietic stem cell transplantation. *Front Immunol*. (2016) 7:188. doi: 10.3389/fimmu.2016.00188
84. Munneke JM, Bjorklund AT, Mjosberg JM, Garming-Legert K, Bernink JH, Blom B, et al. Activated innate lymphoid cells are associated with a reduced susceptibility to graft-versus-host disease. *Blood*. (2014) 124:812–21. doi: 10.1182/blood-2013-11-536888
85. Trabanelli S, Curti A, Lecciso M, Salome B, Riether C, Ochsenbein A, et al. CD127+ innate lymphoid cells are dysregulated in treatment naive acute myeloid leukemia patients at diagnosis. *Haematologica*. (2015) 100:e257–60. doi: 10.3324/haematol.2014.119602
86. Vitale C, Ambrosini P, Montaldo E, Ballerini F, Moretta L, Mingari MC. IL-1β-releasing human acute myeloid leukemia blasts modulate natural killer cell differentiation from CD34+ precursors. *Haematologica*. (2015) 100:e42–5. doi: 10.3324/haematol.2014.110494
87. Dudakov JA, Hanash AM, Jenq RR, Young LF, Ghosh A, Singer NV, et al. Interleukin-22 drives endogenous thymic regeneration in mice. *Science*. (2012) 336:91–5. doi: 10.1126/science.1218004
88. Vely F, Barlogis V, Vallentin B, Neven B, Piperoglou C, Ebbo M, et al. Evidence of innate lymphoid cell redundancy in humans. *Nat Immunol*. (2016) 17:1291–9. doi: 10.1038/ni.3553
89. Moretta F, Petronelli F, Lucarelli B, Pitisci A, Bertaina A, Locatelli F, et al. The generation of human innate lymphoid cells is influenced by the source of hematopoietic stem cells and by the use of G-CSF. *Eur J Immunol*. (2016) 46:1271–8. doi: 10.1002/eji.201546079
90. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity*. (2012) 37:339–50. doi: 10.1016/j.immuni.2012.05.028
91. Lindemans CA, Calafiore M, Mertelsmann AM, O'Connor MH, Dudakov JA, Jenq RR, et al. Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. *Nature*. (2015) 528:560–4. doi: 10.1038/nature16460
92. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol*. (2012) 12:443–58. doi: 10.1038/nri3212
93. Sabat R, Ouyang W, Wolk K. Therapeutic opportunities of the IL-22-IL-22R1 system. *Nat Rev Drug Discov*. (2014) 13:21–38. doi: 10.1038/nrd4176
94. Rankin LC, Girard-Madoux MJ, Seillet C, Mielke LA, Kerdiles Y, Fenis A, et al. Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nat Immunol*. (2016) 17:179–86. doi: 10.1038/ni.3332
95. Roberto A, Castagna L, Gandolfi S, Zanon V, Bramanti S, Sarina B, et al. B-cell reconstitution recapitulates B-cell lymphopoiesis following haploidentical BM transplantation and post-transplant CY. *Bone Marrow Transplant*. (2015) 50:317–9. doi: 10.1038/bmt.2014.266
96. Russo A, Oliveira G, Berglund S, Greco R, Gambacorta V, Cieri N, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood*. (2018) 131:247–62. doi: 10.1182/blood-2017-05-780668
97. Weizman OE, Adams NM, Schuster IS, Krishna C, Pritykin Y, Lau C, et al. ILC1 confer early host protection at initial sites of viral infection. *Cell*. (2017) 171:795–808.e12. doi: 10.1016/j.cell.2017.09.052
98. Vashist N, Trittel S, Ebensen T, Chambers BJ, Guzman CA, Riese P. Influenza-activated ILC1s contribute to antiviral immunity partially influenced by differential GITR expression. *Front Immunol*. (2018) 9:505. doi: 10.3389/fimmu.2018.00505
99. Gorski SA, Hahn YS, Braciale TJ. Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. *PLoS Pathog*. (2013) 9:e1003615. doi: 10.1371/journal.ppat.1003615
100. Duerr CU, McCarthy CD, Mindt BC, Rubio M, Meli AP, Pothlichet J, et al. Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat Immunol*. (2016) 17:65–75. doi: 10.1038/ni.3308
101. Lanier LL. NK cell recognition. *Annu Rev Immunol*. (2005) 23:225–74. doi: 10.1146/annurev.immunol.23.021704.115526
102. Shifrin N, Raulet DH, Ardolino M. NK cell self tolerance, responsiveness and missing self recognition. *Semin Immunol*. (2014) 26:138–44. doi: 10.1016/j.smim.2014.02.007
103. Hudspeth K, Donadon M, Cimino M, Pontarini E, Tentorio P, Preti M, et al. Human liver-resident CD56(bright)/CD16(neg) NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways. *J Autoimm*. (2016) 66:40–50. doi: 10.1016/j.jaut.2015.08.011
104. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science*. (2011) 331:44–9. doi: 10.1126/science.1198687

105. Gupta N, Arthos J, Khazanie P, Steenbeke TD, Censoplano NM, Chung EA, et al. Targeted lysis of HIV-infected cells by natural killer cells armed and triggered by a recombinant immunoglobulin fusion protein: implications for immunotherapy. *Virology*. (2005) 332:491–7. doi: 10.1016/j.virol.2004.12.018
106. Beziat V, Liu LL, Malmberg JA, Ivarsson MA, Sohlberg E, Bjorklund AT, et al. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood*. (2013) 121:2678–88. doi: 10.1182/blood-2012-10-459545
107. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood*. (2002) 100:1935–47. doi: 10.1182/blood-2002-02-0350
108. Brodin P, Hoglund P. Beyond licensing and disarming: a quantitative view on NK-cell education. *Eur J Immunol.* (2008) 38:2934–7. doi: 10.1002/eji.200838760
109. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol.* (2005) 5:201–14. doi: 10.1038/nri1570
110. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*. (1998) 391:795–9. doi: 10.1038/35869
111. Wada H, Matsumoto N, Maenaka K, Suzuki K, Yamamoto K. The inhibitory NK cell receptor CD94/NKG2A and the activating receptor CD94/NKG2C bind the top of HLA-E through mostly shared but partly distinct sets of HLA-E residues. *Eur J Immunol.* (2004) 34:81–90. doi: 10.1002/eji.200324432
112. Campbell KS, Purdy AK. Structure/function of human killer cell immunoglobulin-like receptors: lessons from polymorphisms, evolution, crystal structures and mutations. *Immunology*. (2011) 132:315–25. doi: 10.1111/j.1365-2567.2010.03398.x
113. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, et al. Existence of both inhibitory. (p58) and activatory. (p50) receptors for HLA-C molecules in human natural killer cells. *J Exp Med.* (1995) 182:875–84. doi: 10.1084/jem.182.3.875
114. Manser AR, Weinhold S, Uhrberg M. Human KIR repertoires: shaped by genetic diversity and evolution. *Immunol Rev.* (2015) 267:178–96. doi: 10.1111/imr.12316
115. Zaghi E, Calvi M, Marcenaro E, Mavilio D, Di Vito C. Targeting NKG2A to elucidate natural killer cell ontogenesis and to develop novel immune-therapeutic strategies in cancer therapy. *J Leukoc Biol.* (2019) 105:1243–51. doi: 10.1002/JLB.MR0718-300R
116. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol.* (2002) 169:5118–29. doi: 10.4049/jimmunol.169.9.5118
117. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol.* (2011) 89:216–24. doi: 10.1038/icb.2010.78
118. Kruse PH, Matta J, Ugolini S, Vivier E. Natural cytotoxicity receptors and their ligands. *Immunol Cell Biol.* (2014) 92:221–9. doi: 10.1038/icb.2013.98
119. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev.* (2006) 214:73–91. doi: 10.1111/j.1600-065X.2006.00457.x
120. Di Vito C, Mikulak J, Mavilio D. On the way to become a Natural Killer cell. *Front Immunol.* (2019) 10:1812. doi: 10.3389/fimmu.2019.01812
121. Freud AG, Caligiuri MA. Human natural killer cell development. *Immunol Rev.* (2006) 214:56–72. doi: 10.1111/j.1600-065X.2006.00451.x
122. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol.* (2001) 22:633–40. doi: 10.1016/S1471-4906(01)02060-9
123. Mikulak J, Di Vito C, Zaghi E, Mavilio D. Host immune responses in HIV-1 infection: the emerging pathogenic role of siglecs and their clinical correlates. *Front Immunol.* (2017) 8:314. doi: 10.3389/fimmu.2017.00314
124. Mattiola I, Pesant M, Tentorio PF, Molgora M, Marcenaro E, Lugli E, et al. Priming of human resting NK cells by autologous M1 macrophages via the engagement of IL-1β, IFN-β, and IL-15 pathways. *J Immunol.* (2015) 195:2818–28. doi: 10.4049/jimmunol.1500325
125. Nash WT, Teoh J, Wei H, Gamache A, Brown MG. Know thyself: NK-cell inhibitory receptors prompt self-tolerance, education, and viral control. *Front Immunol.* (2014) 5:175. doi: 10.3389/fimmu.2014.00175
126. Castagna L, Mavilio D. Re-discovering NK cell allo-reactivity in the therapy of solid tumors. *J Immunother Cancer*. (2016) 4:54. doi: 10.1186/s40425-016-0159-4
127. Caligiuri MA. Human natural killer cells. *Blood*. (2008) 112:461–9. doi: 10.1182/blood-2007-09-077438
128. Moretta A. The dialogue between human natural killer cells and dendritic cells. *Curr Opin Immunol.* (2005) 17:306–11. doi: 10.1016/j.coi.2005.03.004
129. Fali T, Papagno L, Bayard C, Mouloud Y, Boddaert J, Sauce D, et al. New insights into lymphocyte differentiation and aging from telomere length and telomerase activity measurements. *J Immunol.* (2019) 202:1962–9. doi: 10.4049/jimmunol.1801475
130. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, et al. CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J Immunol.* (2007) 178:4947–55. doi: 10.4049/jimmunol.178.8.4947
131. Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class. *Immunol Rev*. (2006) 214:143–54. doi: 10.1111/j.1600-065X.2006.00458.x
132. Cichicki F, Schlums H, Theorell J, Tesi B, Miller JS, Ljunggren HG, et al. Diversification and functional specialization of human NK cell subsets. *Curr Top Microbiol Immunol.* (2016) 395:63–94. doi: 10.1007/82_2015_487
133. Hattori N, Saito B, Sasaki Y, Shimada S, Murai S, Abe M, et al. Status of natural killer cell recovery in day 21 bone marrow after allogeneic hematopoietic stem cell transplantation predicts clinical outcome. *Biol Blood Marrow Transplant.* (2018) 24:1841–7. doi: 10.1016/j.bbmt.2018.05.007
134. Minculescu L, Marquart HV, Friis LS, Petersen SL, Schiodt I, Ryder LP, et al. Early natural killer cell reconstitution predicts overall survival in T cell-replete allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2016) 22:2187–93. doi: 10.1016/j.bbmt.2016.09.006
135. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. (2002) 295:2097–100. doi: 10.1126/science.1068440
136. Moretta L, Locatelli F, Pende D, Marcenaro E, Mingari MC, Moretta A, Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *Blood*. (2011) 117:764–71. doi: 10.1182/blood-2010-08-264085
137. Ullah MA, Hill GR, Tey SK. Functional reconstitution of natural killer cells in allogeneic hematopoietic stem cell transplantation. *Front Immunol*. (2016) 7:144. doi: 10.3389/fimmu.2016.00144
138. Pical-Izard C, Crocchiolo R, Granjeaud S, Kochbati E, Just-Landi S, Chabannon C, et al. Reconstitution of natural killer cells in HLA-matched HSCT after reduced-intensity conditioning: impact on clinical outcome. *Biol Blood Marrow Transplant.* (2015) 21:429–39. doi: 10.1016/j.bbmt.2014.11.681
139. Vago L, Forno B, Sormani MP, Crocchiolo R, Zino E, Di Terlizzi S, et al. Temporal, quantitative, and functional characteristics of single-KIR-positive alloreactive natural killer cell recovery account for impaired graft-versus-leukemia activity after haploidentical hematopoietic stem cell transplantation. *Blood*. (2008) 112:3488–99. doi: 10.1182/blood-2007-07-103325
140. Nguyen S, Kuentz M, Vernant JP, Dhedin N, Bories D, Debre P, et al. Involvement of mature donor T cells in the NK cell reconstitution after haploidentical hematopoietic stem-cell transplantation. *Leukemia*. (2008) 22:344–52. doi: 10.1038/sj.leu.2405041
141. Huenecke S, Cappel C, Esser R, Pfirrmann V, Salzmann-Manrique E, Betz S, et al. Development of three different NK cell subpopulations during immune reconstitution after pediatric allogeneic hematopoietic stem cell transplantation: prognostic markers in GvHD and viral infections. *Front Immunol*. (2017) 8:109. doi: 10.3389/fimmu.2017.00109
142. Stabile H, Nisti P, Pagliara D, Locatelli F, Santoni A, Gismondi A. Response to comment on Multifunctional human CD56low CD16low NK cells are the prominent subset in bone marrow of both pediatric healthy donors and leukemic patients. *Haematologica*. (2015) 100:e332–3. doi: 10.3324/haematol.2014.116053
143. Ullrich E, Salzmann-Manrique E, Bakhtiar S, Bremm M, Gerstner S, Herrmann E, et al. Relation between Acute GVHD and NK cell subset

reconstitution following allogeneic stem cell transplantation. *Front Immunol.* (2016) 7:595. doi: 10.3389/fimmu.2016.00595
144. Savani BN, Mielke S, Adams S, Uribe M, Rezvani K, Yong AS, et al. Rapid natural killer cell recovery determines outcome after T-cell-depleted HLA-identical stem cell transplantation in patients with myeloid leukemias but not with acute lymphoblastic leukemia. *Leukemia*. (2007) 21:2145–52. doi: 10.1038/sj.leu.2404892
145. Kim SY, Lee H, Han MS, Shim H, Eom HS, Park B, et al. Post-transplantation natural killer cell count: a predictor of acute graft-versus-host disease and survival outcomes after allogeneic hematopoietic stem cell transplantation. *Clin Lymphoma Myeloma Leuk.* (2016) 16:527–35.e2. doi: 10.1016/j.clml.2016.06.013
146. de Koning C, Plantinga M, Besseling P, Boelens JJ, Nierkens S. Immune reconstitution after allogeneic hematopoietic cell transplantation in children. *Biol Blood Marrow Transplant.* (2016) 22:195–206. doi: 10.1016/j.bbmt.2015.08.028
147. Moretta A, Locatelli F, Moretta L. Human NK cells: from HLA class I-specific killer Ig-like receptors to the therapy of acute leukemias. *Immunol Rev.* (2008) 224:58–69. doi: 10.1111/j.1600-065X.2008.00651.x
148. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood.* (2010) 115:4293–301. doi: 10.1182/blood-2009-05-222190
149. Lang PA, Lang KS, Xu HC, Grusdat M, Parish IA, Recher M, et al. Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. *Proc Natl Acad Sci USA.* (2012) 109:1210–5. doi: 10.1073/pnas.1118834109
150. ChanYLT, Zuo J, Inman C, Croft W, Begum J, Croudace J, et al. NK cells produce high levels of IL-10 early after allogeneic stem cell transplantation and suppress development of acute GVHD. *Eur J Immunol.* (2018) 48:316–29. doi: 10.1002/eji.201747134
151. Simonetta F, Alvarez M, Negrin RS. Natural killer cells in graft-versus-host-disease after allogeneic hematopoietic cell transplantation. *Front Immunol.* (2017) 8:465. doi: 10.3389/fimmu.2017.00465
152. Hu LJ, Zhao XY, Yu XX, Lv M, Han TT, Han W, et al. Quantity and quality reconstitution of NKG2A(+) natural killer cells are associated with graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* (2019) 25:1–11. doi: 10.1016/j.bbmt.2018.08.008
153. Kordelas L, Steckel NK, Horn PA, Beelen DW, Rebmann V. The activating NKG2C receptor is significantly reduced in NK cells after allogeneic stem cell transplantation in patients with severe graft-versus-host disease. *Int J Mol Sci.* (2016) 17:1797. doi: 10.3390/ijms17111797
154. Willem C, Makanga DR, Guillaume T, Maniangou B, Legrand N, Gagne K, et al. Impact of KIR/HLA incompatibilities on NK cell reconstitution and clinical outcome after T cell-replete haploidentical hematopoietic stem cell transplantation with posttransplant cyclophosphamide. *J Immunol.* (2019) 202:2141–52. doi: 10.4049/jimmunol.1801489
155. Ohata K, Espinoza JL, Lu X, Kondo Y, Nakao S. Mycophenolic acid inhibits natural killer cell proliferation and cytotoxic function: a possible disadvantage of including mycophenolate mofetil in the graft-versus-host disease prophylaxis regimen. *Biol Blood Marrow Transplant.* (2011) 17:205–13. doi: 10.1016/j.bbmt.2010.08.014
156. Bremm M, Huenecke S, Zimmermann O, Pfirrmann V, Quaiser A, Bonig H, et al. *In-vitro* influence of mycophenolate mofetil. (MMF) and Ciclosporin A. (CsA) on cytokine induced killer. (CIK) cell immunotherapy. *J Transl Med.* (2016) 14:264. doi: 10.1186/s12967-016-1024-4
157. Orange JS. Human natural killer cell deficiencies and susceptibility to infection. *Microbes Infect.* (2002) 4:1545–58. doi: 10.1016/S1286-4579(02)00038-2
158. De Angelis C, Mancusi A, Ruggeri L, Capanni M, Urbani E, Velardi A, et al. Expansion of CD56-negative, CD16-positive, KIR-expressing natural killer cells after T cell-depleted haploidentical hematopoietic stem cell transplantation. *Acta Haematol.* (2011) 126:13–20. doi: 10.1159/000323661
159. Della Chiesa M, Sivori S, Carlomagno S, Moretta L, Moretta A. Activating KIRs and NKG2C in Viral Infections: toward NK Cell Memory? *Front Immunol.* (2015) 6:573. doi: 10.3389/fimmu.2015.00573
160. Bjorkstrom NK, Ljunggren HG, Sandberg JK. CD56 negative NK cells: origin, function, and role in chronic viral disease. *Trends Immunol.* (2010) 31:401–6. doi: 10.1016/j.it.2010.08.003
161. Mavilio D, Benjamin J, Daucher M, Lombardo G, Kottilil S, Planta MA, et al. Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. *Proc Natl Acad Sci USA.* (2003) 100:15011–6. doi: 10.1073/pnas.2336091100
162. Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, et al. Characterization of CD56-/CD16+ natural killer. (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci USA.* (2005) 102:2886–91. doi: 10.1073/pnas.0409872102
163. Brunetta E, Hudspeth KL, Mavilio D. Pathologic natural killer cell subset redistribution in HIV-1 infection: new insights in pathophysiology and clinical outcomes. *J Leukoc Biol.* (2010) 88:1119–30. doi: 10.1189/jlb.0410225
164. Milush JM, Lopez-Verges S, York VA, Deeks SG, Martin JN, Hecht FM, et al. CD56negCD16(+) NK cells are activated mature NK cells with impaired effector function during HIV-1 infection. *Retrovirology.* (2013) 10:158. doi: 10.1186/1742-4690-10-158
165. Fogli M, Mavilio D, Brunetta E, Varchetta S, Ata K, Roby G, et al. Lysis of endogenously infected CD4+ T cell blasts by rIL-2 activated autologous natural killer cells from HIV-infected viremic individuals. *PLoS Pathog.* (2008) 4:e1000101. doi: 10.1371/journal.ppat.1000101
166. Zhao XY, Luo XY, Yu XX, Zhao XS, Han TT, Chang YJ, et al. Recipient-donor KIR ligand matching prevents CMV reactivation post-haploidentical T cell-replete transplantation. *Br J Haematol.* (2017) 177:766–81. doi: 10.1111/bjh.14622
167. Foley B, Cooley S, Verneris MR, Pitt M, Curtsinger J, Luo X, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood.* (2012) 119:2665–74. doi: 10.1182/blood-2011-10-386995
168. Lopez-Verges S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57(+)NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci USA.* (2011) 108:14725–32. doi: 10.1073/pnas.1110900108
169. Muccio L, Bertaina A, Falco M, Pende D, Meazza R, Lopez-Botet M, et al. Analysis of memory-like natural killer cells in human cytomegalovirus-infected children undergoing αβ+T and B cell-depleted hematopoietic stem cell transplantation for hematological malignancies. *Haematologica.* (2016) 101:371–81. doi: 10.3324/haematol.2015.134155
170. Della Chiesa M, Muccio L, Moretta A. CMV induces rapid NK cell maturation in HSCT recipients. *Immunol Lett.* (2013) 155:11–3. doi: 10.1016/j.imlet.2013.09.020
171. O'Sullivan TE, Sun JC, Lanier LL. Natural killer cell memory. *Immunity.* (2015) 43:634–45. doi: 10.1016/j.immuni.2015.09.013
172. Romee R, Rosario M, Berrien-Elliott MM, Wagner JA, Jewell BA, Schappe T, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med.* (2016) 8:357ra123. doi: 10.1126/scitranslmed.aaf2341
173. Sun JC, Lopez-Verges S, Kim CC, DeRisi JL, Lanier LL. NK cells and immune "memory". *J Immunol.* (2011) 186:1891–7. doi: 10.4049/jimmunol.1003035
174. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science.* (2002) 296:1323–6. doi: 10.1126/science.1070884
175. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood.* (2004) 104:3664–71. doi: 10.1182/blood-2004-05-2058
176. Guma M, Budt M, Saez A, Brckalo T, Hengel H, Angulo A, et al. Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood.* (2006) 107:3624–31. doi: 10.1182/blood-2005-09-3682
177. Hammer Q, Ruckert T, Borst EM, Dunst J, Haubner A, Durek P, et al. Peptide-specific recognition of human cytomegalovirus strains controls adaptive natural killer cells. *Nat Immunol.* (2018) 19:453–63. doi: 10.1038/s41590-018-0082-6
178. Rolle A, Pollmann J, Ewen EM, Le VT, Halenius A, Hengel H, et al. IL-12-producing monocytes and HLA-E control HCMV-driven NKG2C+ NK cell expansion. *J Clin Invest.* (2014) 124:5305–16. doi: 10.1172/JCI77440

179. Charoudeh HN, Terszowski G, Czaja K, Gonzalez A, Schmitter K, Stern M. Modulation of the natural killer cell KIR repertoire by cytomegalovirus infection. *Eur J Immunol.* (2013) 43:480–7. doi: 10.1002/eji.201242389
180. Liu LL, Landskron J, Ask EH, Enqvist M, Sohlberg E, Traherne JA, et al. Critical Role of CD2 Co-stimulation in adaptive natural killer cell responses revealed in NKG2C-deficient humans. *Cell Rep.* (2016) 15:1088–99. doi: 10.1016/j.celrep.2016.04.005
181. Della Chiesa M, Falco M, Bertaina A, Muccio L, Alicata C, Frassoni F, et al. Human cytomegalovirus infection promotes rapid maturation of NK cells expressing activating killer Ig-like receptor in patients transplanted with NKG2C$^{-/-}$ umbilical cord blood. *J Immunol.* (2014) 192:1471–9. doi: 10.4049/jimmunol.1302053
182. Schlums H, Cichocki F, Tesi B, Theorell J, Beziat V, Holmes TD, et al. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity.* (2015) 42:443–56. doi: 10.1016/j.immuni.2015.02.008
183. Zhang T, Scott JM, Hwang I, Kim S. Cutting edge: antibody-dependent memory-like NK cells distinguished by FcRgamma deficiency. *J Immunol.* (2013) 190:1402–6. doi: 10.4049/jimmunol.1203034
184. Lee J, Zhang T, Hwang I, Kim A, Nitschke L, Kim M, et al. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. *Immunity.* (2015) 42:431–42. doi: 10.1016/j.immuni.2015.02.013
185. Gleimer M, von Boehmer H, Kreslavsky T. PLZF controls the expression of a limited number of genes essential for NKT cell function. *Front Immunol.* (2012) 3:374. doi: 10.3389/fimmu.2012.00374
186. Muccio L, Falco M, Bertaina A, Locatelli F, Frassoni F, Sivori S, et al. Late Development of FcepsilonRgamma(neg) adaptive natural killer cells upon human cytomegalovirus reactivation in umbilical cord blood transplantation recipients. *Front Immunol.* (2018) 9:1050. doi: 10.3389/fimmu.2018.01050
187. Luetke-Eversloh M, Hammer Q, Durek P, Nordstrom K, Gasparoni G, Pink M, et al. Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in NKG2Chi natural killer cells. *PLoS Pathog.* (2014) 10:e1004441. doi: 10.1371/journal.ppat.1004441
188. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenschel R, Ditschkowski M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood.* (2011) 118:1402–12. doi: 10.1182/blood-2010-08-304121
189. Green ML, Leisenring WM, Xie H, Walter RB, Mielcarek M, Sandmaier BM, et al. CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood.* (2013) 122:1316–24. doi: 10.1182/blood-2013-02-487074
190. Ito S, Pophali P, Co W, Koklanaris EK, Superata J, Fahle GA, et al. CMV reactivation is associated with a lower incidence of relapse after allo-SCT for CML. *Bone Marrow Transplant.* (2013) 48:1313–6. doi: 10.1038/bmt.2013.49
191. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, et al. Human cytomegalovirus. (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand *in vivo* in response to recipient CMV antigen. *J Immunol.* (2012) 189:5082–8. doi: 10.4049/jimmunol.1201964
192. Jin F, Lin H, Gao S, Wang H, Yan H, Guo J, et al. Characterization of IFNgamma-producing natural killer cells induced by cytomegalovirus reactivation after haploidentical hematopoietic stem cell transplantation. *Oncotarget.* (2017) 8:51–63. doi: 10.18632/oncotarget.13916
193. Capuano C, Pighi C, Battella S, Santoni A, Palmieri G, Galandrini R. Memory NK cell features exploitable in anticancer immunotherapy. *J Immunol Res.* (2019) 2019:8795673. doi: 10.1155/2019/8795673
194. Vantourout P, Hayday A. Six-of-the-best: unique contributions of γδ T cells to immunology. *Nat Rev Immunol.* (2013) 13:88–100. doi: 10.1038/nri3384
195. Pistoia V, Tumino N, Vacca P, Veneziani I, Moretta A, Locatelli F, et al. Human γδ T-cells: from surface receptors to the therapy of high-risk leukemias. *Front Immunol.* (2018) 9:984. doi: 10.3389/fimmu.2018.00984
196. Minculescu L, Sengelov H. The role of gamma delta T cells in haematopoietic stem cell transplantation. *Scand J Immunol.* (2015) 81:459–68. doi: 10.1111/sji.12289
197. Zhao Y, Niu C, Cui J. Gamma-delta (γδ) T cells: friend or foe in cancer development? *J Transl Med.* (2018) 16:3. doi: 10.1186/s12967-018-1491-x
198. Carding SR, Egan PJ. Gammadelta T cells: functional plasticity and heterogeneity. *Nat Rev Immunol.* (2002) 2:336–45. doi: 10.1038/nri797
199. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol.* (2010) 10:467–78. doi: 10.1038/nri2781
200. Braza MS, Klein B, Fiol G, Rossi JF. γδ T-cell killing of primary follicular lymphoma cells is dramatically potentiated by GA101, a type II glycoengineered anti-CD20 monoclonal antibody. *Haematologica.* (2011) 96:400–7. doi: 10.3324/haematol.2010.029520
201. Scheper W, Sebestyen Z, Kuball J. Cancer Immunotherapy Using γδT cells: dealing with diversity. *Front Immunol.* (2014) 5:601. doi: 10.3389/fimmu.2014.00601
202. Adams EJ, Gu S, Luoma AM. Human gamma delta T cells: evolution and ligand recognition. *Cell Immunol.* (2015) 296:31–40. doi: 10.1016/j.cellimm.2015.04.008
203. Kabelitz D, Glatzel A, Wesch D. Antigen recognition by human γδ T lymphocytes. *Int Arch Allergy Immunol.* (2000) 122:1–7. doi: 10.1159/000024353
204. Wesch D, Peters C, Oberg HH, Pietschmann K, Kabelitz D. Modulation of γδ T cell responses by TLR ligands. *Cell Mol Life Sci.* (2011) 68:2357–70. doi: 10.1007/s00018-011-0699-1
205. Scheper W, Grunder C, Straetemans T, Sebestyen Z, Kuball J. Hunting for clinical translation with innate-like immune cells and their receptors. *Leukemia.* (2014) 28:1181–90. doi: 10.1038/leu.2013.378
206. Correia DV, Lopes A, Silva-Santos B. Tumor cell recognition by γδ T lymphocytes: T-cell receptor vs. NK-cell receptors. *Oncoimmunology.* (2013) 2:e22892. doi: 10.4161/onci.22892
207. Correia DV, Fogli M, Hudspeth K, da Silva MG, Mavilio D, Silva-Santos B. Differentiation of human peripheral blood Vdelta1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. *Blood.* (2011) 118:992–1001. doi: 10.1182/blood-2011-02-339135
208. Silva-Santos B, Serre K, Norell H. γδ T cells in cancer. *Nat Rev Immunol.* (2015) 15:683–91. doi: 10.1038/nri3904
209. Bhagat G, Naiyer AJ, Shah JG, Harper J, Jabri B, Wang TC, et al. Small intestinal CD8+TCRγδ+NKG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. *J Clin Invest.* (2008) 118:281–93. doi: 10.1172/JCI30989
210. Todaro M, D'Asaro M, Caccamo N, Iovino F, Francipane MG, Meraviglia S, et al. Efficient killing of human colon cancer stem cells by γδ T lymphocytes. *J Immunol.* (2009) 182:7287–96. doi: 10.4049/jimmunol.0804288
211. Hu Y, Cui Q, Luo C, Luo Y, Shi J, Huang H. A promising sword of tomorrow: human γδ T cell strategies reconcile allo-HSCT complications. *Blood Rev.* (2016) 30:179–88. doi: 10.1016/j.blre.2015.11.002
212. Zheng J, Liu Y, Lau YL, Tu W. γδ-T cells: an unpolished sword in human anti-infection immunity. *Cell Mol Immunol.* (2013) 10:50–7. doi: 10.1038/cmi.2012.43
213. Daniele N, Scerpa MC, Caniglia M, Bernardo ME, Rossi C, Ciammetti C, et al. Transplantation in the onco-hematology field: focus on the manipulation of αβ and γδ T cells. *Pathol Res Pract.* (2012) 208:67–73. doi: 10.1016/j.prp.2011.10.006
214. Handgretinger R, Schilbach K. The potential role of γδ T cells after allogeneic HCT for leukemia. *Blood.* (2018) 131:1063–72. doi: 10.1182/blood-2017-08-752162
215. Perko R, Kang G, Sunkara A, Leung W, Thomas PG, Dallas MH. Gamma delta T cell reconstitution is associated with fewer infections and improved event-free survival after hematopoietic stem cell transplantation for pediatric leukemia. *Biol Blood Marrow Transplant.* (2015) 21:130–6. doi: 10.1016/j.bbmt.2014.09.027
216. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Abhyankar S, et al. Long term disease-free survival in acute leukemia patients recovering with increased γδ T cells after partially mismatched related donor bone marrow transplantation. *Bone Marrow Transplant.* (2007) 39:751–7. doi: 10.1038/sj.bmt.1705650
217. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, et al. HLA-haploidentical stem cell transplantation after removal of αβ+ T and B cells in children with nonmalignant disorders. *Blood.* (2014) 124:822–6. doi: 10.1182/blood-2014-03-563817

218. Hirokawa M, Horiuchi T, Kawabata Y, Kitabayashi A, Miura AB. Reconstitution of γδ T cell repertoire diversity after human allogeneic hematopoietic cell transplantation and the role of peripheral expansion of mature T cell population in the graft. *Bone Marrow Transplant.* (2000) 26:177–85. doi: 10.1038/sj.bmt.1702478
219. Viale M, Ferrini S, Bacigalupo A. TCR gamma/delta positive lymphocytes after allogeneic bone marrow transplantation. *Bone Marrow Transplant.* (1992) 10:249–53.
220. Pabst C, Schirutschke H, Ehninger G, Bornhauser M, Platzbecker U. The graft content of donor T cells expressing gamma delta TCR+ and CD4+foxp3+ predicts the risk of acute graft versus host disease after transplantation of allogeneic peripheral blood stem cells from unrelated donors. *Clin Cancer Res.* (2007) 13:2916–22. doi: 10.1158/1078-0432.CCR-06-2602
221. Lamb LS, Henslee-Downey PJ, Parrish RS, Godder K, Thompson J, Lee C, et al. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. *J Hematother.* (1996) 5:503–9. doi: 10.1089/scd.1.1996.5.503
222. Lamb LS, Gee AP, Hazlett LJ, Musk P, Parrish RS, O'Hanlon TP, et al. Influence of T cell depletion method on circulating γδ T cell reconstitution and potential role in the graft-versus-leukemia effect. *Cytotherapy*. (1999) 1:7–19. doi: 10.1080/00324720310001412 95
223. Scheper W, van Dorp S, Kersting S, Pietersma F, Lindemans C, Hol S, et al. γδT cells elicited by CMV reactivation after allo-SCT cross-recognize CMV and leukemia. *Leukemia*. (2013) 27:1328–38. doi: 10.1038/leu.2012.374
224. Halary F, Pitard V, Dlubek D, Krzysiek R, de la Salle H, Merville P, et al. Shared reactivity of V{delta}2(neg) {gamma}{delta} T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. *J Exp Med.* (2005) 201:1567–78. doi: 10.1084/jem.20041851
225. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood.* (2010) 116:4360–7. doi: 10.1182/blood-2010-01-262089
226. Benson DM, Hofmeister CC, Padmanabhan S, Suvannasankha A, Jagannath S, Abonour R, et al. A phase 1 trial of the anti-KIR antibody IPH2101 in patients with relapsed/refractory multiple myeloma. *Blood*. (2012) 120:4324–33. doi: 10.1182/blood-2012-06-438028
227. Vey N, Bourhis JH, Boissel N, Bordessoule D, Prebet T, Charbonnier A, et al. A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission. *Blood*. (2012) 120:4317–23. doi: 10.1182/blood-2012-06-437558
228. Chiossone L, Vienne M, Kerdiles YM, Vivier E. Natural killer cell immunotherapies against cancer: checkpoint inhibitors and more. *Semin Immunol.* (2017) 31:55–63. doi: 10.1016/j.smim.2017.08.003
229. Muntasell A, Ochoa MC, Cordeiro L, Berraondo P, Lopez-Diaz de Cerio A, Cabo M, et al. Targeting NK-cell checkpoints for cancer immunotherapy. *Curr Opin Immunol.* (2017) 45:73–81. doi: 10.1016/j.coi.2017.01.003
230. Ruggeri L, Urbani E, Andre P, Mancusi A, Tosti A, Topini F, et al. Effects of anti-NKG2A antibody administration on leukemia and normal hematopoietic cells. *Haematologica*. (2016) 101:626–33. doi: 10.3324/haematol.2015.135301
231. Moretta A, Vitale M, Sivori S, Bottino C, Morelli L, Augugliaro R, et al. Human natural killer cell receptors for HLA-class I molecules. Evidence that the Kp43. (CD94) molecule functions as receptor for HLA-B alleles. *J Exp Med.* (1994) 180:545–55. doi: 10.1084/jem.180.2.545
232. McWilliams EM, Mele JM, Cheney C, Timmerman EA, Fiazuddin F, Strattan EJ, et al. Therapeutic CD94/NKG2A blockade improves natural killer cell dysfunction in chronic lymphocytic leukemia. *Oncoimmunology*. (2016) 5:e1226720. doi: 10.1080/2162402X.2016.1226720
233. Godal R, Bachanova V, Gleason M, McCullar V, Yun GH, Cooley S, et al. Natural killer cell killing of acute myelogenous leukemia and acute lymphoblastic leukemia blasts by killer cell immunoglobulin-like receptor-negative natural killer cells after NKG2A and LIR-1 blockade. *Biol Blood Marrow Transplant.* (2010) 16:612–21. doi: 10.1016/j.bbmt.2010.01.019
234. Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res.* (2009) 69:4010–7. doi: 10.1158/0008-5472.CAN-08-3712
235. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. (2000) 408:57–63. doi: 10.1038/35040504
236. Ciurea SO, Schafer JR, Bassett R, Denman CJ, Cao K, Willis D, et al. Phase 1 clinical trial using mbIL21 *ex vivo*-expanded donor-derived NK cells after haploidentical transplantation. *Blood*. (2017) 130:1857–68. doi: 10.1182/blood-2017-05-785659
237. Wang RN, Wen Q, He WT, Yang JH, Zhou CY, Xiong WJ, et al. Optimized protocols for γδ T cell expansion and lentiviral transduction. *Mol Med Rep*. (2019) 19:1471–80. doi: 10.3892/mmr.2019.9831
238. Bertaina A, Zorzoli A, Petretto A, Barbarito G, Inglese E, Merli P, et al. Zoledronic acid boosts γδ T-cell activity in children receiving αβ+ T and CD19(+) cell-depleted grafts from an HLA-haplo-identical donor. *Oncoimmunology*. (2017) 6:e1216291. doi: 10.1080/2162402X.2016.1216291
239. de Witte MA, Sarhan D, Davis Z, Felices M, Vallera DA, Hinderlie P, et al. Early reconstitution of NK and γδ T cells and its implication for the design of post-transplant immunotherapy. *Biol Blood Marrow Transplant.* (2018) 24:1152–62. doi: 10.1016/j.bbmt.2018.02.023
240. Walsh Z, Yang Y, Kohler ME. Immunobiology of chimeric antigen receptor T cells and novel designs. *Immunol Rev.* (2019) 290:100–13. doi: 10.1111/imr.12794
241. Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, et al. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood*. (2011) 118:6050–6. doi: 10.1182/blood-2011-05-354449
242. Ali SA, Shi V, Maric I, Wang M, Stroncek DF, Rose JJ, et al. T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. *Blood*. (2016) 128:1688–700. doi: 10.1182/blood-2016-04-711903
243. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat Med.* (2018) 24:20–8. doi: 10.1038/nm.4441
244. Hay KA, Gauthier J, Hirayama AV, Voutsinas JM, Wu Q, Li D, et al. Factors associated with durable EFS in adult B-cell ALL patients achieving MRD-negative CR after CD19 CAR T-cell therapy. *Blood*. (2019) 133:1652–63. doi: 10.1182/blood-2018-11-883710
245. Klingemann H. Are natural killer cells superior CAR drivers? *Oncoimmunology*. (2014) 3:e28147. doi: 10.4161/onci.28147
246. Hu Y, Tian ZG, Zhang C. Chimeric antigen receptor. (CAR)-transduced natural killer cells in tumor immunotherapy. *Acta Pharmacol Sin.* (2018) 39:167–76. doi: 10.1038/aps.2017.125
247. Mehta RS, Rezvani K. Chimeric antigen receptor expressing natural killer cells for the immunotherapy of cancer. *Front Immunol*. (2018) 9:283. doi: 10.3389/fimmu.2018.00283
248. Di Vito C, Mikulak J, Zaghi E, Pesce S, Marcenaro E, Mavilio D. NK cells to cure cancer. *Semin Immunol.* (2019) 41:101272. doi: 10.1016/j.smim.2019.03.004
249. Rischer M, Pscherer S, Duwe S, Vormoor J, Jurgens H, Rossig C. Human γδ T cells as mediators of chimaeric-receptor redirected anti-tumour immunity. *Br J Haematol.* (2004) 126:583–92. doi: 10.1111/j.1365-2141.2004.05077.x
250. Ingegnere T, Mariotti FR, Pelosi A, Quintarelli C, De Angelis B, Tumino N, et al. Human CAR NK cells: a new non-viral method allowing high efficient transfection and strong tumor cell killing. *Front Immunol*. (2019) 10:957. doi: 10.3389/fimmu.2019.00957
251. Rezvani K. Adoptive cell therapy using engineered natural killer cells. *Bone Marrow Transplant.* (2019) 54:785–8. doi: 10.1038/s41409-019-0601-6
252. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood*. (2005) 106:376–83. doi: 10.1182/blood-2004-12-4797
253. Shimasaki N, Fujisaki H, Cho D, Masselli M, Lockey T, Eldridge P, et al. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. *Cytotherapy.* (2012) 14:830–40. doi: 10.3109/14653249.2012.671519
254. Muller T, Uherek C, Maki G, Chow KU, Schimpf A, Klingemann HG, et al. Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of

lymphoma and leukemia cells. *Cancer Immunol Immunother.* (2008) 57:411–23. doi: 10.1007/s00262-007-0383-3

255. Chu Y, Hochberg J, Yahr A, Ayello J, van de Ven C, Barth M, et al. Targeting CD20+ aggressive B-cell non-hodgkin lymphoma by anti-CD20 CAR mRNA-modified expanded natural killer cells *in vitro* and in NSG mice. *Cancer Immunol Res.* (2015) 3:333–44. doi: 10.1158/2326-6066.CIR-14-0114
256. Tam YK, Maki G, Miyagawa B, Hennemann B, Tonn T, Klingemann HG. Characterization of genetically altered, interleukin 2-independent natural killer cell lines suitable for adoptive cellular immunotherapy. *Hum Gene Ther.* (1999) 10:1359–73. doi: 10.1089/10430349950018030
257. Jochems C, Hodge JW, Fantini M, Fujii R, Morillon YM II, Greiner JW, et al. An NK cell line. (haNK) expressing high levels of granzyme and engineered to express the high affinity CD16 allele. *Oncotarget.* (2016) 7:86359–73. doi: 10.18632/oncotarget.13411
258. Liu E, Tong Y, Dotti G, Shaim H, Savoldo B, Mukherjee M, et al. Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. *Leukemia.* (2018) 32:520–31. doi: 10.1038/leu.2017.226
259. Oei VYS, Siernicka M, Graczyk-Jarzynka A, Hoel HJ, Yang W, Palacios D, et al. Intrinsic functional potential of NK-cell subsets constrains retargeting driven by chimeric antigen receptors. *Cancer Immunol Res.* (2018) 6:467–80. doi: 10.1158/2326-6066.CIR-17-0207
260. Mirzaei HR, Mirzaei H, Lee SY, Hadjati J, Till BG. Prospects for chimeric antigen receptor. (CAR) γδ T cells: a potential game changer for adoptive T cell cancer immunotherapy. *Cancer Lett.* (2016) 380:413–23. doi: 10.1016/j.canlet.2016.07.001
261. van der Veken LT, Hagedoorn RS, van Loenen MM, Willemze R, Falkenburg JH, Heemskerk MH. Alphabeta T-cell receptor engineered γδ T cells mediate effective antileukemic reactivity. *Cancer Res.* (2006) 66:3331–7. doi: 10.1158/0008-5472.CAN-05-4190

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REVIEW
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Mechanisms of Graft-versus-Host Disease Prevention by Post-transplantation Cyclophosphamide: An Evolving Understanding

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Post-transplantation cyclophosphamide (PTCy) has been highly successful at preventing severe acute and chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic cell transplantation (HCT). The clinical application of this approach was based on extensive studies in major histocompatibility complex (MHC)-matched murine skin allografting models, in which cyclophosphamide was believed to act via three main mechanisms: (1) selective elimination of alloreactive T cells; (2) intrathymic clonal deletion of alloreactive T-cell precursors; and (3) induction of suppressor T cells. In these models, cyclophosphamide was only effective in very specific contexts, requiring particular cell dose, cell source, PTCy dose, and recipient age. Achievement of transient mixed chimerism also was required. Furthermore, these studies showed differences in the impact of cyclophosphamide on transplanted cells (tumor) versus tissue (skin grafts), including the ability of cyclophosphamide to prevent rejection of the former but not the latter after MHC-mismatched transplants. Yet, clinically PTCy has demonstrated efficacy in MHC-matched or partially-MHC-mismatched HCT across a wide array of patients and HCT platforms. Importantly, clinically significant acute GVHD occurs frequently after PTCy, inconsistent with alloreactive T-cell elimination, whereas PTCy is most active against severe acute GVHD and chronic GVHD. These differences between murine skin allografting and clinical HCT suggest that the above-mentioned mechanisms may not be responsible for GVHD prevention by PTCy. Indeed, recent work by our group in murine HCT has shown that PTCy does not eliminate alloreactive T cells nor is the thymus necessary for PTCy's efficacy. Instead, other mechanisms appear to be playing important roles, including: (1) reduction of alloreactive $CD4^+$ effector T-cell proliferation; (2) induced functional impairment of surviving alloreactive $CD4^+$ and $CD8^+$ effector T cells; and (3) preferential recovery of $CD4^+$ regulatory T cells. Herein, we review the history of cyclophosphamide's use in preventing murine skin allograft rejection and our evolving new understanding of the mechanisms underlying its efficacy in preventing GVHD after HCT. Efforts are ongoing

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to more fully refine and elaborate this proposed new working model. The completion of this effort will provide critical insight relevant for the rational design of novel approaches to improve outcomes for PTCy-treated patients and for the induction of tolerance in other clinical contexts.

Keywords: post-transplantation cyclophosphamide, haploidentical allogeneic hematopoietic cell transplantation, graft-versus-host disease, skin allograft rejection, alloreactive T cells, tolerance, regulatory T cells

INTRODUCTION

Hematopoietic cell transplantation (HCT) is the only potentially curative treatment for many patients with advanced hematologic malignancies or severe non-malignant diseases. Human leukocyte antigen (HLA)-matched donors are the historical gold standard source for HCT, but they are unavailable for many patients, including the majority of those not of white European ethnicity (1). The use of donors who are not fully HLA-matched has been associated with high levels of graft-versus-host disease (GVHD) and graft rejection (2, 3), which are attributable to strong bi-directional responses of donor and host alloreactive T cells (3, 4). Fortunately, newer strategies have improved outcomes for HCT using partially HLA-mismatched donors, resulting in outcomes comparable with those seen with HLA-matched donor HCT (5).

Among these strategies, post-transplantation cyclophosphamide (PTCy) has become widely used as GVHD prophylaxis (6). A research group from Johns Hopkins University was the first to utilize high-dose PTCy in their platform for HLA-haploidentical bone marrow HCT (7, 8); PTCy since has been extended to other HCT platforms and a variety of donor types (6, 9). Accelerating its widespread adoption is the fact that PTCy is an inexpensive treatment that does not require extensive training for its administration, thus resulting in high accessibility. Furthermore, clinical outcomes using PTCy have been quite promising; registry data suggest that PTCy reduces chronic GVHD incidence after HLA-haploidentical HCT, resulting in decreased GVHD rates but similar survival compared with patients undergoing standard HCT using HLA-matched related or unrelated donors (10–13). The benefits of PTCy in preventing severe acute and chronic GVHD observed in the HLA-haploidentical HCT setting also have been found in the HLA-matched donor HCT setting (14–16), further decreasing the need for other post-transplant immunosuppression (17).

Recently, in a prospective multi-center, randomized phase II clinical trial, Bolaños-Meade and colleagues have compared the relative efficacy of three novel approaches for GVHD prophylaxis after reduced-intensity conditioning, HLA-matched related or unrelated donor HCT (18). Each of the three novel agents was added to standard calcineurin-inhibitor (CNI)-based GVHD prophylaxis, and each regimen was compared to a contemporaneous non-randomized control group receiving standard tacrolimus and methotrexate (18). Only the PTCy-containing regimen resulted in superior GVHD-free, relapse-free survival (the primary endpoint). The PTCy-containing regimen also provided superior protection against both severe acute GVHD and chronic GVHD requiring immunosuppressive therapy (18).

While we still await the results of ongoing and planned randomized phase III studies, PTCy already has had a dramatic impact on the HCT field. Rates of HLA-haploidentical HCT have risen precipitously, and the use of PTCy steadily has been making in-roads into HLA-matched HCT, which will likely accelerate given the results from the randomized phase II study detailed above. Yet, in order to rationally improve upon outcomes seen with HCT using PTCy, it is of the utmost importance to have a detailed understanding of the mechanisms by which PTCy prevents GVHD after HCT. It has been long believed that we understood these mechanisms based on extrapolation from major histocompatibility complex (MHC)-matched murine skin allografting models using cyclophosphamide (19, 20). However, these mechanisms have not fit with some of the clinical observations in human HCT (e.g., no substantial impact of PTCy on grade II acute GVHD), and recent data suggest that these mechanistic explanations may not be true in murine HCT models (21–23). Therefore, this review provides an overview of the history of cyclophosphamide's use in preventing skin allografting rejection experimentally and of the evolving understanding regarding mechanisms of GVHD prevention by PTCy after HCT.

EARLY STUDIES OF CYCLOPHOSPHAMIDE AS A TOLEROGENIC AGENT

There is an intriguing duality in the history of cyclophosphamide as it has been used both as a pro-inflammatory and as a tolerogenic agent (24). The latter has taken many forms experimentally, including prolongation of skin allograft survival, mitigation of GVHD, and prevention of antibody-mediated responses to antigens, such as erythrocytes (25–35).

In the early 1960s, Berenbaum and Brown began to investigate the use of cyclophosphamide to prolong skin allograft survival in an MHC-haploidentical murine experimental model. They observed that a single high-dose injection of cyclophosphamide given during the critical window of days 0 to +4 was able to delay skin allograft rejection (25). Owens and Santos found in a partially-MHC-mismatched murine HCT model that when cyclophosphamide was given at 37.5 or 75 mg/kg/day on days +5, +8, +11, and +14, it was successful at preventing fatal GVHD, distinct from 6-mercaptopurine, methotrexate, cortisol, or mechlorethamine, which were all ineffective (33). Although protected from fatal GVHD, cyclophosphamide-treated mice still rejected skin allografts from the host or third-party

strains, but with slower kinetics than untreated mice, suggesting that alloreactivity against host antigens was not lost after cyclophosphamide. Moreover, in a rat HCT model, low-dose (5–10 mg/kg/day) cyclophosphamide was successful at preventing fatal GVHD when given on days +2, +3, and +5, but was ineffective if begun later, starting on day +7 (35). Overall, these several studies showed that PTCy could exert a tolerogenic effect on allograft survival or GVHD when given early post-transplant.

In an attempt to improve murine skin allograft survival, Nirmul and colleagues (1973) introduced a new concept in which they gave priming allogeneic MHC-haploidentical splenocytes on day 0, cyclophosphamide on day +2, and skin allografts (from the same donor strain as the splenocytes) on day +12 (34). This approach produced a 2-week prolongation of skin allograft survival, although these grafts still were rejected (34). These investigators also began to more thoroughly investigate the timing and dosage of cyclophosphamide, observing that a 200 mg/kg single dose on day +2 was superior in effect to the same total dose divided in smaller doses over days +2 to +5. They also found that the dose of splenocytes administered was important, as a dose of 100 x 10^6 cells was more effective than 50 $\times$ 10^6 cells (34).

EFFICACY OF CYCLOPHOSPHAMIDE IN PREVENTING REJECTION OF MHC-MATCHED SKIN ALLOGRAFTS

In a series of 13 papers from 1984 to 1987, a Japanese investigative group from Kyushu University continued to study and develop the approach pioneered by Nirmul and colleagues. At first, they evaluated the differences between tumor and skin allograft survival in models like Nirmul's, in which viable donor splenocytes were given prior to cyclophosphamide administration. Consistent with previous data on the optimal timing of cyclophosphamide in skin allografting models (34), MHC-mismatched tumors continued to grow if cyclophosphamide was given between 1 and 3 days after splenocyte administration, whereas accelerated rejection was seen if cyclophosphamide was given on day −2, 0, +5, or +7 in relation to splenocyte administration (36). Tolerance was antigen-specific, however, as injection of tumors of a different MHC-mismatched strain were rejected even with cyclophosphamide. Furthermore, prevention of tumor rejection required giving viable donor splenocytes at a specific dose, as simply infusing large amounts of soluble donor antigen was ineffective (36).

Surprisingly, MHC-mismatched skin allografts would be rejected in models in which MHC-mismatched tumors were not rejected, suggesting critical differences between responses to the two types of allografts (36). Furthermore, the dose of MHC-mismatched tumor given to cyclophosphamide-treated mice was important as small doses were rejected rapidly, while large doses of tumor were not rejected (37). Despite continued tumor growth, mice treated with cyclophosphamide retained antigen-specific cytotoxic lymphocyte responses to tumor alloantigen after cyclophosphamide as measured via delayed footpad reaction and chromium release cytotoxicity assays, and there actually was heightened reactivity early after cyclophosphamide treatment (38).

However, a very different effect was seen after MHC-matched skin allografting. In this setting, survival of skin allografts was substantially prolonged in cyclophosphamide-treated mice, which was accompanied by abrogated responses in delayed footpad reactions and cytotoxicity assays (39). Prolonged skin allograft survival also was seen in syngeneic sex-mismatched transplants, in which male skin allografts were not rejected by female hosts when primed with male splenocytes beforehand and given cyclophosphamide on day 0 (40). Other differences were observed between the MHC-matched and MHC-mismatched systems. Thymectomy had no effect in MHC-mismatched models (41), but resulted in decreased skin allograft survival in MHC-matched models (42). Conversely, splenectomy could prolong skin allograft survival somewhat in the MHC-mismatched models but had no effect in MHC-matched models (42).

This group spent a considerable amount of time better developing and understanding their model system. They found that the activity of cyclophosphamide in MHC-matched skin allografting peaked when given on days +2 or +3 after infusion of the priming splenocytes, as measured by skin allograft survival, delayed footpad reactions, and cytotoxicity assays (43). The cyclophosphamide dose seemed important as it was marginally less effective when given at 150 mg/kg than at 200 mg/kg (43). The dose and type of cells also were important; if thymocytes or bone marrow cells were used as the priming cells, cyclophosphamide was much less effective (43, 44). Yet, the presence of viable donor stem cells either from spleens or bone marrow was necessary for successful maintenance of MHC-matched skin allograft, and some degree of donor engraftment and mixed chimerism in the thymus was critical (44, 45). Irradiating the priming donor cells abrogated the tolerogenic effect, and lower doses of cells actually led to accelerated rejection (44). The age of the recipient, but not the donor, was crucial; there was no significant difference between recipients of 6–12 weeks of age, but reduced tolerance to the allograft was observed when recipient mice were 40 weeks of age (46). Thus, the development of this model identified several crucial parameters needed for successful prolongation of skin allograft survival by cyclophosphamide, including MHC-matched donor/recipient pairs, specific dose and timing of PTCy, specific dose and type of priming donor cells, achievement of intrathymic mixed chimerism, and specific age of the recipient.

Later efforts were focused on trying to overcome the barrier to MHC-mismatched skin allografting. Two successful approaches were identified. The first consisted of T-cell depletion with anti-Thy1.2 antibody treatment on day −1, transplant of spleen and bone marrow cells on day 0, followed by high-dose cyclophosphamide on day +2 (47). Another method successful in overcoming the barrier to MHC-mismatched skin allografting involved two rounds of cyclophosphamide, which could successfully first induce tolerance across major MHC antigens and then secondarily across minor antigens (48).

PROPOSED MECHANISMS BY WHICH CYCLOPHOSPHAMIDE PREVENTED REJECTION OF MHC-MATCHED SKIN ALLOGRAFTS

This group proposed that three mechanisms mediate prevention of MHC-matched skin allograft rejection by cyclophosphamide (49). The first mechanism, thought to be the dominant one, was described as direct deletion by cyclophosphamide of highly proliferating host mature T cells that were alloreactive to donor antigens. The second mechanism was proposed to be intrathymic clonal deletion of donor-alloreactive host precursor T cells, and the third mechanism was proposed to be induction of host suppressor T cells (49).

Regarding the first mechanism, the authors showed that in the MHC-matched setting, splenocytes derived from tolerized mice at day +35 were unresponsive *in vitro* to stimulation from cells from the priming strain, whereas they responded normally to third-party antigens (50). They hypothesized that this unresponsiveness was due to selective elimination of alloreactive T cells by cyclophosphamide. To test this hypothesis, they leveraged mismatches within the minor lymphocyte stimulating system [responses to proviruses of the mouse mammary tumor virus incorporated into the genomes of certain mouse strains (51)] between different mouse strains to provide markers of alloreactive T cells. In their MHC-matched models, mixed chimerism was established in the lymph nodes by day +14 (49). At that time point in the lymph nodes, there was a substantial two-thirds reduction in the percentages of $CD4^+$ T cells, but not $CD8^+$ T cells, that were donor-alloreactive ($V\beta6^+$); there was continued decline through days +35 and +70 in the percentage of $CD4^+$ T cells in the lymph nodes that were $V\beta6^+$ (49, 50, 52), although a small but detectable (10% of original percentage) population of $V\beta6^+CD4^+$ T cells remained. However, the percentages of donor-alloreactive $V\beta6^+CD4^+$ T cells both in the lymph nodes and in the thymus (see below) increased again by day +100. Additionally, studies of host-alloreactive donor $V\beta3^+CD4^+$ T cells in one of the MHC-matched models showed a decline in their percentages within $CD4^+$ T cells in the lymph nodes at day +10, although there was persistent mixed chimerism in these mice (53).

Regarding the second proposed mechanism, intrathymic clonal deletion, the investigators found in the thymus that donor-alloreactive $V\beta6^+CD4^+$ T cells remained at normal levels at day +14 after cyclophosphamide, at which point there was minimal intrathymic donor chimerism (49, 52). However, $V\beta6^+CD4^+$ T-cell percentages steadily declined thereafter such that they were quite low by day +35 (49, 52), at which point there was low but easily detectable donor chimerism in the thymus. Surprisingly in some mice, donor-alloreactive $V\beta6^+CD4^+$ T cells began to reappear in the thymus at day +70 to +100, which corresponded with loss of substantive intrathymic donor chimerism (49). Interestingly, this loss of donor-alloreactive T-cell intrathymic clonal deletion was not associated with skin allograft rejection (49). Thus, the authors concluded that intrathymic clonal deletion of donor-alloreactive T-cell precursors did occur after cyclophosphamide and required intrathymic mixed chimerism, but was not essential for maintenance of skin allografts at late stages.

The third proposed mechanism, induction of host suppressor T cells, was thought to be the least important of the three and only active at late time points (49). These investigators found that transferring splenocytes at day +14 from tolerized mice to new irradiated mice led to only a short prolongation of skin allograft survival (49). After 100 days, however, transferring splenocytes in this fashion led to marked prolongation of skin allografts (49). This latter effect also was true in models using mice mismatched at both major and minor histocompatibility antigens, even in the absence of any mixed chimerism (54). In their MHC-matched models, antibody treatment to deplete all T cells or just $CD4^+$ or $CD8^+$ cells suggested that $CD8^+$ regulatory T cells were the cells responsible for this effect, since depleting T cells in general or $CD8^+$ cells led to more rapid skin allograft rejection (49, 54). However, later work contradicted these findings and instead showed that T-cell or $CD4^+$-cell depletion (but not $CD8^+$-cell depletion) would obviate the suppressive activity, suggesting instead that $CD4^+$ regulatory T cells were responsible (55). These $CD4^+$ regulatory T cells mediated suppression in cyclophosphamide-treated mice in an alloantigen-specific manner, which was important since they also showed that donor-alloreactive effector T cells did persist and were not anergic (55).

In their proposed model, there were important interactions between the three mechanisms. As described above, direct peripheral elimination of alloreactive T cells was thought to be the dominant mechanism, followed by intrathymic clonal deletion of alloreactive T-cell precursors. The authors concluded that intrathymic mixed chimerism was necessary for this second step to occur, but intrathymic clonal deletion terminated once mixed chimerism ended. At that turning point, occurring in some mice between days +35 and +100 (49), suppressor T cells became most critical, maintaining a state of immunologic tolerance and preventing skin allograft rejection (49, 54). Playing an adjunct role to suppression, clonal anergy also was suggested to contribute to long-lasting tolerance, since the authors observed no proliferative response in donor-alloreactive T cells that reappeared in the periphery once intrathymic clonal deletion ended (49, 56); however, a similar lack of response also was seen in mice primed with allogeneic MHC-matched splenocytes without cyclophosphamide (49). Furthermore, later findings also showed persistence of donor-alloreactive T cells with the ability to respond to alloantigen and thus not anergic (55), suggesting that the model and its dynamics may be even more complex than initially described.

POTENTIAL LIMITATIONS OF THIS MECHANISTIC MODEL

Other investigators at the University of Pittsburgh and Johns Hopkins drew on this rich immunologic background to examine the impact of cyclophosphamide given post-transplant in preventing GVHD in HCT models (57–60). This has led directly

to the successful clinical translation of this approach in both HCT and in combined solid organ/hematopoietic cell transplantation (6–8, 61, 62).

Yet, the mechanistic model (20) used to explain how cyclophosphamide works clinically largely has been extrapolated from the MHC-matched skin allografting models reviewed above, in which the efficacy of cyclophosphamide was contextual. For cyclophosphamide to prevent skin allograft rejection, the host had to be 6–12 weeks old (46), the transplant had to be MHC-matched (36, 39), a specific dose and type of priming cells had to be used (43), the priming cells had to contain viable stem cells (44), the dose of cyclophosphamide had to be 150–200 mg/kg (43, 44), and a minimal level of mixed chimerism had to be achieved, even if only transiently (45, 52). A single round of cyclophosphamide was unable to overcome the MHC barrier, but instead a two-step approach or inclusion of T-cell-depleting antibodies had to be performed (47, 48).

These models showed that cyclophosphamide led to a decline in the frequency of alloreactive T cells only in the MHC-matched setting. In the MHC-mismatched setting, an initial decline in alloreactive T-cell percentages at day +14 was followed by subsequent rising percentages over the next 3 weeks. The authors conjectured, but did not demonstrate, that highly proliferative T-cell clones would be sensitive to cyclophosphamide, resulting in their elimination, and consequent tolerance induction; conversely, slowly proliferative clones were hypothesized to survive cyclophosphamide treatment in a sensitized state, and thus no tolerance would be induced (63). The authors interpreted the findings of inability of a single dose of cyclophosphamide to induce tolerance to MHC-mismatched skin allografts and selectively eliminate alloreactive T cells as indicating that alloreactivity in the MHC-mismatched setting has slower kinetics than in the MHC-matched setting; in this framework, T cells responding to MHC-mismatched antigens would be less sensitive to cyclophosphamide (39). Such a conclusion is at odds with our broader immunologic understanding that alloreactive T-cell responses are more potent in the MHC-mismatched setting, including clinical manifestations of intense and rapid alloreactivity in the HLA-haploidentical setting that are not observed after HLA-matched HCT (64–66).

Furthermore, the decline in the percentages of donor-alloreactive T cells seen in MHC-matched skin allografting models steadily progressed over several weeks instead of immediately after cyclophosphamide. These kinetics suggest that peripheral deletional tolerance may be operational and may be acting in addition to or instead of direct deletion by cyclophosphamide. Transferring splenocytes at day +14 from tolerized mice to newly irradiated mice led to only minimal prolongation of skin allograft survival, confirming that alloreactive T cells survived cyclophosphamide and retained some functionality (49). Moreover, the reduction in the percentages of alloreactive T cells was shown to be transient and associated with a resurgence of alloreactive T cells that displayed functional impairment (49, 50, 53, 56). Indeed, persistent alloreactive T cells present at 10 weeks after cyclophosphamide could provoke graft rejection in particular circumstances (55). Similarly, splenocytes taken from mice tolerized by PTCy could cause GVHD, inconsistent with the hypothesis of selective alloreactive T-cell elimination. When splenocytes from C3H mice tolerized by cyclophosphamide were given as donor cells for HCT into the same strain used for the priming splenocytes (AKR), no GVHD was induced. But, use of the reverse model (AKR→C3H) resulted in chronic GVHD reactions (67). Likewise, fatal GVHD occurred, albeit at a slightly delayed interval, when cyclophosphamide-treated cells from MHC-mismatched donor/recipient combinations were used (67). Overall, decreases in alloreactive T cells in lymph nodes were observed in some contexts after cyclophosphamide, but were not shown to be due to direct destruction by cyclophosphamide nor were they directly linked mechanistically to prevention of skin allograft rejection.

Although the investigators demonstrated that intrathymic clonal deletion occurred, this also was not strongly linked experimentally with prevention of skin allograft rejection, but rather correlated with mixed chimerism. Thymectomy could worsen outcomes in this model, but half of thymectomized mice still maintained the graft long-term. Moreover, when it occurred in thymectomized mice, rejection generally happened later (42), and thymectomy only had an impact in MHC-matched models (41, 42). Notably, intrathymic clonal deletion could break down at later stages and tolerance to the skin allograft would still be maintained (49), drawing into question the importance of this mechanism for prevention of skin allograft rejection. Also, in some model systems, intrathymic mixed chimerism was not necessary (54). Finally, the role of regulatory T cells was established but there was discrepancy between studies on the relative role of $CD4^+$ vs. $CD8^+$ regulatory T cells (49, 55).

Ultimately, it is unclear whether some of the elements of the model are mechanistic vs. epiphenomena. Moreover, it is unknown what findings are specific for cyclophosphamide's effects only in the context of this specific model vs. being broadly applicable to other contexts. This is of particular importance given the differential effects seen in these skin allografting models between transplantation of cells vs. solid organs and between MHC-matched vs. MHC-mismatched allografts and the fact that cyclophosphamide has proven efficacy clinically for partially HLA-mismatched HCT (6, 9).

TOWARD A NEW UNDERSTANDING

Despite the limitations noted above, a mechanistic model largely based on extrapolation from the skin allografting models was developed to explain how PTCy prevents GVHD (20, 68); this mechanistic model has since become entrenched in the HCT field. Yet, there are important inconsistencies between what would be predicted from this model and what actually has been observed clinically after HCT. PTCy is most effective at preventing chronic GVHD and the progression of moderate (grade II) to severe (grade III-IV) acute GVHD (6). However, grade II acute GVHD frequently occurs in PTCy-treated patients at rates of 20–80% (6, 15, 16, 69, 70), which would suggest that alloreactive T cells persist after PTCy and are not anergic. Nonetheless, only 10–20% of patients develop chronic GVHD

(6, 15, 16, 69, 70), indicating no ongoing clinical alloreactivity even though alloreactive T cells may persist. Furthermore, PTCy is broadly effective in HCT patients across an array of recipient ages, cell doses, HLA-matching, and PTCy doses (6, 9, 69, 71–73), contrasting with the specific requirements needed in the MHC-matched skin allografting studies. Lastly, treatment with a CNI prior to cyclophosphamide blocked cyclophosphamide's efficacy in the skin allografting models (74), but clinically CNIs can be integrated prior to PTCy without any loss, and potentially even improvement, in prevention of GVHD after HCT (75, 76). Therefore, we began to question how well the proposed mechanistic model extrapolated from the MHC-matched skin allografting models (20, 49) serves as an accurate depiction of how PTCy prevents GVHD after allogeneic HCT.

In the skin allografting models, it was posited that regulatory T cells only serve a later role to compensate for breakdown of clonal deletion and reemergence of alloreactive T cells. Yet, given the apparent persistence of alloreactive T cells clinically, we explored the role of $CD4^+$ regulatory T cells (Tregs) in HCT in two studies. First, we found that $CD4^+$ Tregs recovered rapidly after HCT in patients despite protracted total $CD4^+$ T-cell lymphopenia (21). Additionally, we observed that human $CD4^+$ Tregs were more resistant to PTCy in mixed lymphocyte cultures *in vitro* and increased expression of aldehyde dehydrogenase (ALDH), the major *in vivo* detoxifying enzyme for cyclophosphamide, both *in vivo* and *in vitro* (21). Beyond just better survival and reconstitution, $CD4^+$ Tregs were necessary early post-transplant for GVHD prevention by PTCy in both xenogeneic and MHC-matched HCT models (21, 22), and thymically-derived natural $CD4^+Foxp3^+$ Tregs, rather than peripherally induced $CD4^+Foxp3^+$ Tregs, appeared to be playing the major role in the murine MHC-matched HCT models (22). These findings overall solidified a role for Tregs that seemed more critical than previously ascribed and more important at earlier time points. Notably, given that Tregs were necessary immediately post-HCT for GVHD prevention by PTCy (21, 22), these results implied that alloreactive T-cell elimination either was not occurring or was not as complete after PTCy as had been previously believed.

Given the primary clinical use of PTCy for HLA-haploidentical HCT, we next developed an MHC-haploidentical HCT model (B6C3F1→B6D2F1) that parallels clinical HCT to further study the mechanisms underlying GVHD prevention by PTCy (23). We used this model to clarify the three previously proposed mechanisms of GVHD prevention by PTCy: selective elimination of alloreactive T cells, intrathymic clonal deletion of alloreactive T-cell precursors, and induction of Tregs (20, 49). Our primary goal was to test the hypothesis that alloreactive T-cell elimination is a necessary and central mechanism of GVHD prevention by PTCy. In this model, we administered PTCy on days +3 and +4 to further parallel clinical HCT. We first established the optimal PTCy dose (25 mg/kg/day) for GVHD prevention in our model; either lower or higher doses of PTCy were less effective at preventing GVHD and mortality. Alloreactive T cells robustly proliferated post-transplant, consistent with results seen in human HLA-haploidentical HCT (77, 78). $CD4^+$ T-cell proliferation was reduced but not eliminated after 25 mg/kg/day PTCy (**Figure 1**). Surprisingly, $CD8^+$ T-cell proliferation was not substantially reduced by 25 mg/kg/day PTCy (**Figure 1**).

Using $V\beta6^+$ as a marker of host-alloreactive donor T cells (akin to what was done in the skin allografting models), we observed that host-alloreactive donor T cells persisted at percentages similar to or even higher than seen in mice not treated with PTCy. The persistence of $V\beta6^+$ host-alloreactive donor T cells after PTCy was demonstrated in this model at days +7, +21, and +200 and at specific time points in three other murine HCT models: at day +7 in another MHC-haploidentical model (B6→B6D2F1), at days +6 and +200 in an MHC-mismatched model (C3H→B6D2F1), and at day +7 in an MHC-matched model (C3H→AKR), and the persistence of $V\beta3^+$ and $V\beta5^+$ host-alloreactive donor T cells was seen at day +7 in a third MHC-haploidentical model (B6→B6C3F1) (23).

To additionally confirm that PTCy was not selectively eliminating alloreactive T cells, two other markers of host-alloreactive donor T cells were used: 2C T-cell receptor $(TCR)^+$ $CD8^+$ T cells and 4C TCR^+ $CD4^+$ T cells. 2C TCR^+ T cells from B6C3F1 [(C3H x 2C TCR^+ B6)F1] mice were admixed with wild-type T cells to generate allografts containing a fixed percentage (8%) of the $CD8^+$ T cells having the 2C TCR; splenocytes containing this mixture were used as the donor cells for HCT. A similar approach was used for studying 4C TCR^+ T cells as a percentage (8%) of $CD4^+$ T cells. 2C TCR^+ $CD8^+$ host-alloreactive donor T cells remained highly proliferative despite PTCy and actually expanded from 8% of donor $CD8^+$ T cells at transplant up to 30–80% of donor $CD8^+$ T cells by day +7 (**Figure 1**) and still were detectable at day +200 (23). Yet, PTCy remained effective at GVHD prevention in this model. 4C TCR^+ T cells also persisted after PTCy at similar to higher percentages as in vehicle-treated mice, but their proliferation after PTCy was reduced, as we had seen for $V\beta6^+$ host-alloreactive donor $CD4^+$ T cells (23). In these studies, it was not possible to definitively separate the relative resistance of different T-cell subsets to PTCy vs. changes in early reconstitution after PTCy, particularly given how rapidly host-alloreactive donor T cells were proliferating despite PTCy (and thus numerically expanding) and the *in vivo* system that limited exhaustive tracking of whole-body alloreactive T-cell numbers; regardless, host-alloreactive donor T cells were not selectively eliminated, and if anything, appeared to dominate early reconstitution after PTCy (**Figure 1**).

To test the fate of host-non-alloreactive donor T cells after HCT, we used our admixed 2C TCR^+ approach in an MHC-haploidentical model B6→B6C3F1, wherein 2C TCR^+ T cells are host-non-alloreactive. In this setting, 2C TCR^+ T cells maintained a naïve phenotype and low-level proliferation (**Figure 1**) (23). Thus, since host-alloreactive donor T cells continued to proliferate rapidly and expand despite PTCy, the percentage of $CD8^+$ T cells that were host-non-alloreactive 2C TCR^+ actually greatly contracted (**Figure 1**). These results demonstrated that GVHD prevention could be achieved by PTCy despite persistence and even expansion of host-alloreactive donor T cells, whereas host-non-alloreactive donor T cells appear not to be the dominant population early after PTCy (**Figure 1**) as had been previously believed (20). This differs from the previously

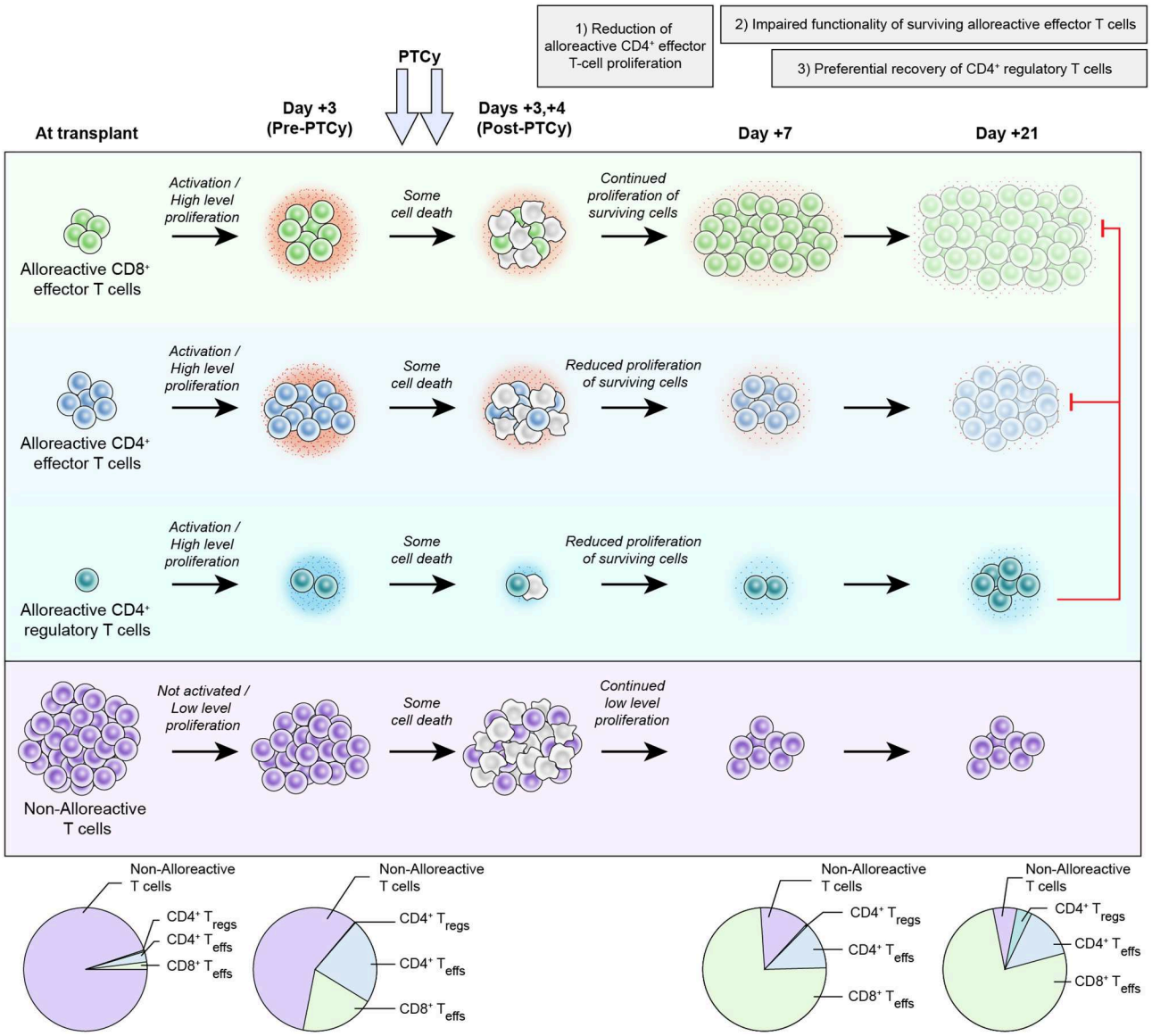

FIGURE 1 | Proposed new working model of the mechanisms by which post-transplantation cyclophosphamide prevents graft-versus-host disease after allogeneic hematopoietic cell transplantation. After infusion into the recipient, host-alloreactive donor T cells become activated, highly proliferative, and more productive of inflammatory cytokines (depicted by the small red dots around the cells). Since PTCy, as an alkylator, is not a cell cycle-dependent cytotoxic agent, both host-alloreactive donor T cells (highly proliferative), and host-non-alloreactive donor T cells (lowly proliferative) are affected and there is some cell death in all subsets. It is unclear *in vivo* whether host-alloreactive donor T cells are more resistant to PTCy or just more rapidly reconstitute due to their ongoing rapid proliferation; survival (or death) after PTCy likely is due to a complex array of factors occurring in each individual cell and may be modulated by several interrelated factors (24). Between post-transplant days +3 and +7, there is continued high-level proliferation of host-alloreactive donor $CD8^+$ effector T cells and reduced but continued proliferation of the surviving host-alloreactive donor $CD4^+$ T cells (effector (Teff) and regulatory (Treg) cells). The reduced proliferation of host-alloreactive donor $CD4^+$ effector T cells seems to be important for PTCy's efficacy as dosing schedules that fail to decrease host-alloreactive donor $CD4^+$ effector T-cell proliferation are ineffective or suboptimal at preventing GVHD (79). Impaired functionality of surviving host-alloreactive donor effector T cells occurs early after PTCy (as early as post-transplant day +5) and appears to increase with time, as depicted with progressive decreases in cytokine production and changes in color of those subsets. This change in host-alloreactive donor effector T-cell functionality appears to be in part a direct (or at least rapid) effect of PTCy, but also is augmented by preferential reconstitution of donor $CD4^+$ regulatory T cells between days +7 and +21, which suppress the host-alloreactive donor effector T cells, further contributing to their impaired functionality. Throughout this entire process, host-non-alloreactive donor T cells are not activated, maintain low-level proliferation, and consequently contract relative to host-alloreactive donor T cells. The dynamics of host-alloreactive vs. host-non-alloreactive donor T cells at later time points are unknown, but indirect data suggest that they may change with time and/or antigenic (e.g., viral) stimulation (80). This proposed working model is heavily based on experimental data in murine HCT (21–23) and does not account for the integration of other immunosuppressant agents with PTCy as is commonly done clinically. However, the proposed model is expected to be imperfect and incomplete, and further study likely will increase its accuracy, comprehensiveness, and complexity.

proposed model wherein antigen-activated donor T cells with high proliferation were proposed to be preferentially targeted by PTCy, whereas non-alloreactive donor T cells were believed to be much less affected, leading to an immune reconstitution devoid of alloreactive T cells (20).

If host-alloreactive donor T cells persist and even expand after PTCy, how is GVHD being mitigated? We examined this question in two different ways (23). The first approach isolated liver-infiltrating donor cells from mice treated with either vehicle or 25 mg/kg/day PTCy and re-stimulated them *in vitro* with alloantigen. We found that PTCy-treated T cells continued to proliferate, but did so to a lesser degree than vehicle-treated mice. Although the effect of PTCy on proliferation was more modest, PTCy-treated cells also produced dramatically less inflammatory cytokines (**Figure 1**). Similar findings of decreased proliferation and cytokine production were seen when using donor T cells that were flow cytometrically devoid of $CD4^+CD25^+$ T cells to isolate the effect on host-alloreactive donor T cells themselves, rather than the confounding effect of the presence of donor Tregs in the cultures. These results suggested that PTCy was leading to intrinsic functional impairment of host-alloreactive donor T cells (**Figure 1**). We confirmed these findings by using our 2C TCR^+ admixed model described above, treating mice with vehicle or 25 mg/kg/day PTCy on days +3 and +4, flow cytometrically sorting viable 2C TCR^+ $CD8^+$ T cells on day +5, and reinfusing them into new irradiated mice which were not further treated. In these serial transplants, mice transplanted with PTCy-treated 2C TCR^+ T cells had better weights and clinical scores than mice transplanted with vehicle-treated 2C TCR^+ T cells, despite similar persistence of 2C TCR^+ T cells numerically at day +150. These results confirmed that PTCy appears to be rapidly inducing functional impairment of surviving alloreactive T cells that contributes to GVHD prevention (**Figure 1**). Although the nature of this functional impairment has not yet been fully characterized, both the *in vitro* and *in vivo* data above showed that host-alloreactive donor T cells treated with PTCy do continue to respond to alloantigens, albeit at a reduced level, suggesting that they are not becoming anergic.

The second mechanism previously proposed to underlie PTCy's efficacy in preventing skin allograft rejection was intrathymic clonal deletion of alloreactive T-cell precursors. However, in our MHC-haploidentical HCT model (B6C3F1→B6D2F1), we demonstrated that 25 mg/kg/day PTCy on days +3 and +4 remained effective in thymectomized recipients with no apparent difference in outcomes between mice that were or were not thymectomized (23). These results disproved that the thymus plays a necessary role in GVHD prevention by PTCy. Furthermore, PTCy-treated mice transplanted in these models quickly converted to full donor chimeras (23), demonstrating that mixed chimerism is not necessary to achieve GVHD prevention by PTCy.

The third mechanism previously proposed was the induction of suppressor T cells (49, 54). Our previous data showed that $CD4^+Foxp3^+$ Tregs rapidly reconstituted in HCT patients by a month after PTCy, preferentially survived PTCy *in vitro*, and were necessary for GVHD prevention immediately after PTCy in xenogeneic and MHC-matched HCT models (21, 22). We further investigated the impact of $CD4^+CD25^+Foxp3^+$ Tregs in our MHC-haploidentical model. We were surprised to find that the percentages of donor $CD4^+$ T cells that were $CD25^+Foxp3^+$ were similar or lower at day +7 (after PTCy on days +3/+4) compared with vehicle-treated mice (23); these results were different from what we had observed in human or mouse *in vitro* mixed lymphocyte cultures (21, 22), in which the percentages of Tregs were increased at day +7. Even so, in our MHC-haploidentical HCT model, donor $CD4^+CD25^+Foxp3^+$ Tregs preferentially reconstituted by day +21 in all four tested organs in mice treated with 25 mg/kg/day PTCy on days +3 and +4 (**Figure 1**) (23). This effect appeared dose-dependent as mice treated with too low (5 mg/kg/day) or too high (100 mg/kg/day) PTCy doses did not have preferential donor Treg recovery. The lack of preferential recovery of Tregs after the 100 mg/kg/day PTCy dose was not due to an inadequate recovery of Tregs, as the numbers were similar with 25 mg/kg/day PTCy-treated mice, but rather due to a much more robust rebound of host-alloreactive donor effector T cells after 100 mg/kg/day PTCy; these findings may explain the worse GVHD seen histopathologically with that higher dose (23). Thus, the ability of PTCy to constrain host-alloreactive donor effector T-cell proliferation seemed to be optimal after the 25 mg/kg/day dose on days +3 and +4.

Our results provided a few additional novel insights into the role of donor Tregs in GVHD prevention by PTCy. Alloantigen-specific donor Tregs were increased in the liver of PTCy-treated mice at day +21 compared with vehicle-treated mice, and the Treg content of the liver at day +50 correlated very well with the histopathologic score of GVHD in those organs (23). Furthermore, $Foxp3^+$ Treg depletion in our MHC-haploidentical model (B6C3F1→B6D2F1) demonstrated a time dependency. Although they were necessary early post-transplant as we also had shown in our other models (21–23), donor Tregs appeared to play an increasingly important role as time progressed post-transplant; much higher mortality and more rapid and severe GVHD induction were observed when Tregs were depleted at later time points (day +30 and especially day +60 or +150) (23). We also explored whether donor Tregs were sufficient to prevent GVHD in our model as they were in MHC-matched murine HCT or human T-cell-depleted HLA-haploidentical HCT (81, 82). We tested this in our T-cell-replete MHC-haploidentical HCT model by giving $CD4^+CD25^+$ donor Tregs 4 days prior to HCT; GVHD lethality was delayed, but ultimately these mice still developed severe and fatal GVHD, suggesting that donor Tregs, while necessary for GVHD prevention by PTCy, may not be sufficient to prevent GVHD after T-cell-replete MHC-haploidentical HCT (23).

We further tested the role of suppressive mechanisms in GVHD prevention by PTCy in another set of experiments in which mice were transplanted with our B6C3F1→B6D2F1 MHC-haploidentical HCT model and at a later date had new donor splenocytes infused. Consistent with our findings that PTCy does not work via alloreactive T-cell elimination and that suppressive mechanisms are critical, infusion of new donor splenocytes at various time points (day +5, day +28, day +126, day +150, day +200) did not cause GVHD (23). In fact, mice reinfused on days +5 or +28 were indistinguishable from mice

not reinfused with new donor splenocytes. These results suggest that suppressive mechanisms are induced immediately after PTCy and are sufficient to prevent new donor T cells from causing GVHD. We are actively exploring the relevant role of donor Tregs vs. other suppressive cell populations in mediating this effect and whether there may be other immunosuppressive players involved in GVHD prevention by PTCy. Thus, the model proposed in **Figure 1** may really serve as a starting place from which to build and refine a complete mechanistic model of GVHD prevention by PTCy. This goal is a major focus of ongoing investigations in our laboratory.

As we increasingly have come to recognize that the previous paradigm of understanding for how cyclophosphamide prolongs MHC-matched skin allograft survival may not apply to GVHD prevention by PTCy, we also have begun to question what we thought we knew about how PTCy should best be applied clinically. As we have described above, the maximal efficacy of PTCy in the MHC-matched skin allografting models was achieved with a high dose (200 mg/kg) given on day +2 or +3 (43). Murine HCT models, building on the skin allografting data, used the 200 mg/kg dose on day +2 or +3 to decrease graft rejection and GVHD (57–60). When PTCy then was translated to patients (8), it was given at 50 mg/kg [close to the maximum tolerable dose in humans and a dose which had showed efficacy in aplastic anemia treatment (83)] and was given on day +3 to space it as far away from conditioning as possible aiming to decrease toxicity. Results from the first phase II study suggested that adding a second dose of PTCy on day +4 might lead to less extensive chronic GVHD than dosing on day +3 only (7); thus, nearly all subsequent studies have used dosing of PTCy at 50 mg/kg/day on days +3 and +4. Our studies in murine MHC-haploidentical and MHC-mismatched HCT models showed that an intermediate dose of PTCy of 25 mg/kg/day on days +3 and +4 appeared superior to both lower and higher doses (23). Consequently, we have extensively studied the impact of the timing and dosing of PTCy on its efficacy in preventing GVHD in our murine MHC-haploidentical HCT model (79). We found that the dose, timing, and cumulative exposure of PTCy all impacted substantially on how well it prevented GVHD and that there were interactions between these three parameters (79). Ultimately, the peak efficacy of PTCy appeared to be at approximately day +4, with dosing at earlier or later time points being less effective; this finding was most pronounced when using suboptimal doses of PTCy (79). Furthermore, the most effective dosing schema of PTCy both reduced host-alloreactive ($V\beta6^+$) donor $CD4^+CD25^-Foxp3^-$ effector T-cell proliferation at day +7 and allowed preferential donor $CD4^+CD25^+Foxp3^+$ regulatory T-cell reconstitution at day +21, which together may serve as potential biomarkers of effective GVHD prevention by PTCy (79). Compared with PTCy dosing on days +3/+4, dosing on days +1/+2 did not reduce host-alloreactive donor $CD4^+$ effector T-cell proliferation at day +7 as effectively, while dosing on days +5/+6 or +7/+8 hindered preferential donor Treg recovery at day +21 (79). Based on these data, we are currently exploring in a clinical trial whether the dosing and timing of PTCy can be optimized in HLA-haploidentical HCT (NCT03983850) with the goals of further improving hematopoietic and immune reconstitution after HCT and reducing toxicity, while maintaining or potentially even improving prevention of acute and chronic GVHD.

IMMUNOLOGIC INSIGHTS FROM CLINICAL HCT

As described earlier, we have shown that activated Tregs reconstitute rapidly in patients post-transplant, recovering close to donor levels within a month after HCT despite prolonged $CD4^+$ T-cell lymphopenia (21). Another group showed that higher percentages of Tregs at day +14 were associated with decreased risk for acute GVHD (84), while we showed that the percentages of Tregs at days +30 and +60 actually were elevated in patients with active acute GVHD (21), suggesting a potential compensatory increase in those patients. However, these studies have focused on Tregs as a bulk population, and alloantigen-specific Tregs have not been studied in HCT patients treated with PTCy.

The identification of alloreactive effector or regulatory T cells in humans is complicated. Unlike in mice, where we can know that a specific T-cell clone is alloreactive in a specific setting, in humans we generally have to rely on functional characteristics associated with a cell to prove it as an alloreactive clone. Alloreactive T cells are generally expected to derive largely from the naïve T-cell pool, wherein each clone would be expected to exist as a single cell or only a few cells. Thus, it can be difficult to track the fate of alloreactive T cells post-transplant due simply to the inadequacy of sampling; indeed, clones that are <0.01% of T cells cannot be reliably detected on repeat sampling even from the same sample that is split into two halves for T-cell receptor sequencing (80). Furthermore, apparent alloreactive T-cell clones found within GVHD target tissues are not always found in the blood of the same patients (80, 85).

Even so, we recently studied immune reconstitution by flow cytometry and TCR sequencing in patients treated with HLA-matched HCT using PTCy as single-agent GVHD prophylaxis (80). Despite complete or near-complete donor chimerism (15), surprisingly, the TCR repertoires in patients at 1 month post-transplant bore little resemblance to their donors' TCR repertoires (80). In fact, T-cell clones that were expanded in donors were generally undetectable in recipients at 1–2 months post-transplant, whereas frequent donor T-cell clones in patients at 1–2 months post-transplant were generally not able to be tracked back to their origin within the donor repertoires (80); importantly, these patients were older and had been heavily pre-treated, with minimal recent thymic emigrants at these time points. This implied that the repertoire early post-transplant indeed may be largely derived from rare, presumably naïve, clones found in the donor. Indeed, other groups have reported that T-cell reconstitution early after PTCy appears to be coming predominantly from naïve-derived T cells that assume a stem-cell-memory-like phenotype (77, 78). Conversely, memory T-cell clones in the donor, particularly those reactive to pathogens like cytomegalovirus, were not found at high levels early post-transplant, but began to dominate the T-cell repertoire

after 3 months post-transplant, at which time point the TCR repertoire in the recipient became increasingly similar to the donor (80). Overall, these results in patients are completely consistent with the new proposed working model (**Figure 1**), in which expansion of alloreactive T cells, derived in patients from rare donor T cells, occurs early post-transplant despite PTCy. However, the human studies have not yet linked these naïve-derived T-cell clones present early post-transplant to be specifically alloreactive.

Although T cells have been the primary focus of these studies and the proposed mechanistic model, the impact of PTCy on other immune cells has been investigated. Both B cell and natural killer (NK) cells appear largely to turn over post-transplant, as the cells that do recover tend to be predominantly naïve mature B cells and immature NK cells (80, 86). However, there are no data available regarding any potential role of either of these subsets in acute or chronic GVHD prevention by PTCy. In patients treated with PTCy and bortezomib for GVHD prophylaxis, dendritic cells isolated early post-transplant had decreased expression of co-stimulatory and maturation markers (87). T cells stimulated with these dendritic cells were less proliferative than T cells stimulated with dendritic cells derived from patients treated with standard CNI-based GVHD prophylaxis (87). However, it is unclear what of this effect is due to PTCy vs. bortezomib and whether this is mechanistic or an epiphenomenon.

IMMUNOLOGIC INSIGHTS FROM CLINICAL SOLID ORGAN TRANSPLANTATION

The success of clinical solid organ transplantation is limited by absence of tolerance induction in the vast majority of patients, generally requiring long-term immunosuppression to prevent allograft loss. As an alternative, investigators have pursued the addition of HCT to solid organ transplantation with the goal of inducing long-term tolerance and thus the ability to reduce or remove immunosuppression. Given PTCy's efficacy clinically in HCT and preclinically in skin and other solid organ transplantation models (19, 57, 58, 88), PTCy has been incorporated into some approaches to HLA-mismatched or HLA-haploidentical combined HCT/kidney transplantation (61, 62, 89, 90); the results have been promising with very low rates of GVHD and the ability to fully remove immunosuppression in all patients with persisting donor chimerism except for one patient with chronic GVHD (89, 90). However, in patients without durable donor chimerism, graft rejection could occur even when hyporesponsiveness of recipient cells to donor cells *in vitro* was observed (62). These data suggest that mixed chimerism was protective, but that the T cells present after PTCy still could mediate graft rejection.

Morris and colleagues have proposed an *in vitro* method to screen for donor-alloreactive T cells, wherein mixed lymphocyte reactions were performed between donor and recipient PBMCs, followed by flow cytometric sorting of the T cells reactive to the donor antigens (91). Deep sequencing of the TCRβ repertoires was performed in order to identify the presumptive alloreactive T cells (those proliferating in response to alloantigen) and evaluate the fate of these clones after transplant. Six patients were studied. Even in PTCy-treated patients who were functionally tolerant, anti-donor T-cell responses could be seen persisting for 6–18 months post-transplant. Although a decrease in donor-reactive T-cell clones was observed post-transplant, overall the reduction was modest and progressive over 6–18 months post-transplant (91). This did differ from the two patients studied who were not treated with PTCy, in which progressive increases in the number of donor-reactive $CD4^+$ T-cell clones were seen. Overall, these results support that alloreactive T-cell deletion may be occurring to a limited extent in patients after combined HCT/kidney transplantation using PTCy. Yet, this does not appear to be an immediate effect of PTCy but rather a progressive change over months to years, reflecting likely peripheral deletional tolerance in these mixed chimeric states.

DISCUSSION

The use of cyclophosphamide for inducing tolerance to skin allografting models had been thought to rest on three principles, with the primary mechanism being selective elimination of alloreactive T cells by PTCy (49, 53). Although a preferential reduction of alloreactive T cells over time was shown in MHC-matched skin allografting models, it was not shown in MHC-mismatched models. Furthermore, the slow reduction of alloreactive T-cell percentages after cyclophosphamide was not directly linked to killing by cyclophosphamide. Minimal levels of mixed chimerism were an essential component that consistently tracked with alloreactive T-cell depletion in those studies. Indeed, we have observed peripheral deletion of alloreactive T cells in thymectomized mice treated with T-cell-depleted HCT without PTCy (23). Thus, it is unclear whether the reduction of alloreactive T cells seen in the MHC-matched skin allografting models was directly or indirectly related to cyclophosphamide. Even if that effect was directly related to cyclophosphamide, the effectiveness of cyclophosphamide in those models was contextual, including differential effects on tumor vs. skin allografts and on MHC-matched vs. MHC-mismatched allografts, raising concerns about the relevance of such a model for MHC-haploidentical HCT (**Table 1**). Indeed, clinically PTCy is highly effective across an array of donor types, transplant platforms, graft compositions and cell doses, and recipient and donor ages (6). In our recent paper (23), we tried to replicate some of the contextuality of the skin allografting models by exploring different doses of PTCy, the inclusion or exclusion of radiation prior to HCT, and investigation of MHC-matched, MHC-haploidentical, and fully MHC-mismatched models, but in all cases we saw persistence of host-alloreactive donor T cells in the recipients at percentages that were similar to or even higher than were transplanted (23).

Our current understanding of how PTCy works to prevent GVHD has greatly evolved over the past several years. The initial proposed mechanistic model extrapolated from the skin allografting models had posited that PTCy works

TABLE 1 | Elements of the previously proposed mechanistic model as relates to outcomes observed with experimental murine skin allografting or allogeneic HCT.

	Skin grafting	Hematopoietic cell transplantation
Alloreactive T cells	• The percentages of donor-alloreactive $CD4^+$ T cells were selectively reduced between days 0 and +35 in MHC-matched models, increasing again afterwards. This was associated with abrogation of alloreactive functional responses. • Host-alloreactive donor $CD4^+$ T cells were selectively reduced at day +10 in MHC-matched models. • There was not a sustained decrease in donor-alloreactive $CD4^+$ T cells in MHC-mismatched models. This was associated with persistence and early increases in alloreactive functional responses. • The reduction of donor-alloreactive $CD4^+$ T cells in MHC-matched models was not linked mechanistically with the fate of skin allografts. • Donor-alloreactive $CD4^+$ T cells present at later time points were in many cases anergic *in vitro*; when not anergic, they still had reduced functionality. Even so, these alloreactive T cells retained sufficient functionality to cause graft rejection or GVHD in serial transplants and also could cause graft rejection when regulatory T cells were removed.	• No selective elimination of host-alloreactive donor T cells was observed at early or late time points. • The lack of host-alloreactive donor T-cell elimination was seen in MHC-matched, MHC-haploidentical, and MHC-mismatched HCT models. • Persistence of host-alloreactive donor T cells was seen regardless of the dose of PTCy used or whether the mice were irradiated. • GVHD prevention occurred despite host-alloreactive donor T-cell persistence. • PTCy at the optimal dose had minimal impact on host-alloreactive donor $CD8^+$ T-cell proliferation but did reduce host-alloreactive donor $CD4^+$ T-cell proliferation. • 2C TCR^+ donor T cells preferentially expanded despite PTCy in a model wherein they were host-alloreactive, but contracted in a model wherein they were host-non-alloreactive. Thus, host-alloreactive donor T cells appeared to preferentially reconstitute post-transplant due to their survival and continued proliferation after PTCy. • Host-alloreactive donor T cells did have reduced functionality after PTCy in terms of their *in vitro* proliferation and cytokine production in response to alloantigen and their *in vivo* ability to cause GVHD in that mouse or on serial transfer. Yet, these alloreactive T cells were not functionally or phenotypically anergic. • Host-alloreactive donor T-cell elimination was seen in thymectomized mice treated with T-cell-depleted HCT without PTCy, suggesting that peripheral deletion of alloreactive T cells can occur independently of PTCy.
Intrathymic clonal deletion of alloreactive T-cell precursors	• Intrathymic clonal deletion occurred only in settings wherein cyclophosphamide was effective, but was not linked mechanistically with skin graft rejection. • Intrathymic clonal deletion was linked in most cases with intrathymic mixed chimerism that was at least transient in nature. • Thymectomy decreased survival of skin allografts in a subset of mice in MHC-matched models, but had no impact in MHC-mismatched models. • The breakdown of intrathymic clonal deletion was associated with loss of intrathymic mixed chimerism, but skin allografts were not rejected when this occurred.	• The thymus was not necessary for GVHD prevention by PTCy. • Outcomes were similar between thymectomized and non-thymectomized mice treated with PTCy. • Full donor chimerism was rapidly achieved in PTCy-treated mice. Of note, mice treated with TCD BM only had persistent mixed chimerism within T cells despite the myeloablative conditioning intensity.
Suppressor cells	• Depletion of suppressor T cells at late time points reduced allograft survival. There were mixed data regarding whether depletion of $CD4^+$ or $CD8^+$ T cells mediated this effect. • The transfer of splenocytes at day +14 from mice tolerized by cyclophosphamide only slightly prolonged skin allograft survival, whereas it led to a much greater prolongation of survival when performed at day +100. • The suppression exerted by $CD4^+$ Tregs was mediated in an alloantigen-specific manner.	• $CD4^+CD25^+Foxp3^+$ donor T cells, including those that were alloantigen-specific, preferentially reconstituted after PTCy. • $CD4^+CD25^+Foxp3^+$ donor T cells played a necessary role in GVHD prevention by PTCy, but did not appear sufficient to prevent severe and fatal GVHD. • $Foxp3^+$ donor T cells were necessary for GVHD prevention by PTCy both at early and late post-transplant time points, but appeared increasingly important as time progressed. • The suppressive mechanisms induced early after PTCy were sufficient to prevent new donor T cells from causing GVHD.

primarily through selectively eliminating alloreactive T cells (68). Subsequently, our work in showing a necessary role for Tregs confirmed the role of Tregs identified in the skin allografting models; therefore, the model was revised to include the preferential recovery of Tregs and an important balance between effector and regulatory T cells (20). Additionally, intrathymic clonal deletion was added back into the model to reflect the initial MHC-matched skin allografting data (20, 49). Our recent work has affirmed the role of Tregs, but showed that neither alloreactive T-cell elimination nor intrathymic clonal elimination are necessary for GVHD prevention by PTCy (23). Rather, PTCy appears to induce functional impairment of alloreactive T cells, which is supported by the rapid induction of active suppressive mechanisms after PTCy. These suppressive mechanisms include the preferential recovery of Tregs, including those that are alloantigen-specific. Ongoing work in the laboratory suggests that the model may be even more complicated than that shown in **Figure 1**, but the presented working model at least displays our level of understanding at this time.

This current understanding fits much better with clinical observations after PTCy than the prior model. PTCy is effective in older and heavily pretreated patients, many of whom lack substantive thymic function (6). Clinically, acute GVHD occurs frequently after PTCy and actually is associated with improved outcomes in those patients (69, 92), consistent with persistence of alloreactive T cells after PTCy. Yet, severe acute or chronic GVHD is infrequent after PTCy, consistent with our model that persisting alloreactive T cells are becoming functionally impaired. GVHD incidence is associated with lower levels of Tregs (23, 84), but clinically Tregs also have been found to be increased in patients with active acute GVHD (21); therefore, Tregs may play roles both in preventing acute GVHD but also controlling breakthrough GVHD and preventing its progression to more severe forms. However, how the integration of adjunct immunosuppression with PTCy, both in terms of the agents used and their timing in relation to PTCy, affects GVHD prevention by PTCy and PTCy's impact on specific immune subsets require further study (93). Furthermore, our recently published work suggests that intermediate, rather than high, dose PTCy may be most effective at preventing GVHD in two murine HCT models (23). Additionally, we have recently demonstrated in our murine MHC-haploidentical HCT model that the optimal timing of PTCy appears to differ from that demonstrated in murine skin allografting models (79). The clinical relevance of these findings concerning the optimal dosing and timing of PTCy requires further study in human HCT, which is being done in a clinical study at our institution.

Improving upon outcomes of patients treated with PTCy may be achieved most rapidly by using a paradigm of understanding that is both based on HCT data and consistent with clinical observations in humans. The persistence of alloreactive T cells after PTCy may allow for the reinduction of a graft-versus-host anti-tumor response in patients with relapsed or aggressive disease post-transplant. Conversely, better understanding the highly active suppressive mechanisms induced by PTCy may allow for better prevention and treatment of GVHD and other post-transplant inflammatory conditions. These mechanisms will provide insight into the pathophysiology of GVHD and its prevention, but also may be directly relevant for improving outcomes in other clinical settings in which tolerance induction is desired, such as autoimmunity and solid organ transplantation. Ultimately, we hope that our recent data and proposed working model will help facilitate the rational development of novel approaches to further reduce GVHD incidence and severity, improve immune reconstitution, and decrease malignancy relapse post-transplant.

AUTHOR CONTRIBUTIONS

NN and CK wrote the manuscript and designed the figure.

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REFERENCES

1. Gragert L, Eapen M, Williams E, Freeman J, Spellman S, Baitty R, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med.* (2014) 371:339–48. doi: 10.1056/NEJMsa1311707
2. Anasetti C, Amos D, Beatty PG, Appelbaum FR, Bensinger W, Buckner CD, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med.* (1989) 320:197–204. doi: 10.1056/NEJM198901263200401
3. Anasetti C, Beatty PG, Storb R, Martin PJ, Mori M, Sanders JE, et al. Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. *Hum Immunol.* (1990) 29:79–91. doi: 10.1016/0198-8859(90)90071-V
4. DeWolf S, Sykes M. Alloimmune T cells in transplantation. *J Clin Invest.* (2017) 127:2473–81. doi: 10.1172/JCI90595
5. Kanakry CG, de Lima MJ, Luznik L. Alternative donor allogeneic hematopoietic cell transplantation for acute myeloid leukemia. *Semin Hematol.* (2015) 52:232–42. doi: 10.1053/j.seminhematol.2015.03.005
6. Kanakry CG, Fuchs EJ, Luznik L. Modern approaches to HLA-haploidentical blood or marrow transplantation. *Nat Rev Clin Oncol.* (2016) 13:10–24. doi: 10.1038/nrclinonc.2015.128
7. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using non-myeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
8. O'Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, Phelps M, et al. Non-myeloablative bone marrow transplantation from partially HLA-mismatched related donors using posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2002) 8:377–86. doi: 10.1053/bbmt.2002.v8.pm12171484
9. Kasamon YL, Ambinder RF, Fuchs EJ, Zahurak M, Rosner GL, Bolanos-Meade J, et al. Prospective study of nonmyeloablative, HLA-mismatched unrelated BMT with high-dose posttransplantation cyclophosphamide. *Blood Adv.* (2017) 1:288–92. doi: 10.1182/bloodadvances.2016002766
10. Kanate AS, Mussetti A, Kharfan-Dabaja MA, Ahn KW, DiGilio A, Beitinjaneh A, et al. Reduced-intensity transplantation for lymphomas using

haploidentical related donors vs HLA-matched unrelated donors. *Blood.* (2016) 127:938–47. doi: 10.1182/blood-2015-09-671834
11. Ghosh N, Karmali R, Rocha V, Ahn KW, DiGilio A, Hari PN, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors versus HLA-matched sibling donors: a center for international blood and marrow transplant research analysis. *J Clin Oncol.* (2016) 34:3141–9. doi: 10.1200/JCO.2015.66.3476
12. Dreger P, Sureda A, Ahn KW, Eapen M, Litovich C, Finel H, et al. PTCy-based haploidentical vs matched related or unrelated donor reduced-intensity conditioning transplant for DLBCL. *Blood Adv.* (2019) 3:360–9. doi: 10.1182/bloodadvances.2018027748
13. Ciurea SO, Zhang MJ, Bacigalupo AA, Bashey A, Appelbaum FR, Aljitawi OS, et al. Haploidentical transplant with posttransplant cyclophosphamide vs. matched unrelated donor transplant for acute myeloid leukemia. *Blood.* (2015) 126:1033–40. doi: 10.1182/blood-2015-04-639831
14. Luznik L, Bolanos-Meade J, Zahurak M, Chen AR, Smith BD, Brodsky R, et al. High-dose cyclophosphamide as single-agent, short-course prophylaxis of graft-versus-host disease. *Blood.* (2010) 115:3224–30. doi: 10.1182/blood-2009-11-251595
15. Kanakry CG, O'Donnell PV, Furlong T, de Lima MJ, Wei W, Medeot M, et al. Multi-institutional study of post-transplantation cyclophosphamide as single-agent graft-versus-host disease prophylaxis after allogeneic bone marrow transplantation using myeloablative busulfan and fludarabine conditioning. *J Clin Oncol.* (2014) 32:3497–505. doi: 10.1200/JCO.2013.54.0625
16. Kanakry CG, Tsai HL, Bolanos-Meade J, Smith BD, Gojo I, Kanakry JA, et al. Single-agent GVHD prophylaxis with posttransplantation cyclophosphamide after myeloablative, HLA-matched BMT for AML, ALL, and MDS. *Blood.* (2014) 124:3817–27. doi: 10.1182/blood-2014-07-587477
17. Kanakry CG, Bolanos-Meade J, Kasamon YL, Zahurak M, Durakovic N, Furlong T, et al. Low immunosuppressive burden after HLA-matched related or unrelated BMT using posttransplantation cyclophosphamide. *Blood.* (2017) 129:1389–93. doi: 10.1182/blood-2016-09-737825
18. Bolanos-Meade J, Reshef R, Fraser R, Fei M, Abhyankar S, Al-Kadhimi Z, et al. Three prophylaxis regimens (tacrolimus, mycophenolate mofetil, and cyclophosphamide; tacrolimus, methotrexate, and bortezomib; or tacrolimus, methotrexate, and maraviroc) versus tacrolimus and methotrexate for prevention of graft-versus-host disease with haemopoietic cell transplantation with reduced-intensity conditioning: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). *Lancet Haematol.* (2019) 6:e132–43. doi: 10.1016/S2352-3026(18)30221-7
19. Mayumi H, Umesue M, Nomoto K. Cyclophosphamide-induced immunological tolerance: an overview. *Immunobiology.* (1996) 195:129–39. doi: 10.1016/S0171-2985(96)80033-7
20. Luznik L, O'Donnell PV, Fuchs EJ. Post-transplantation cyclophosphamide for tolerance induction in HLA-haploidentical bone marrow transplantation. *Semin Oncol.* (2012) 39:683–93. doi: 10.1053/j.seminoncol.2012.09.005
21. Kanakry CG, Ganguly S, Zahurak M, Bolanos-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. *Sci Transl Med.* (2013) 5:211ra157. doi: 10.1126/scitranslmed.3006960
22. Ganguly S, Ross DB, Panoskaltsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, et al. Donor CD4+ Foxp3+ regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. *Blood.* (2014) 124:2131–41. doi: 10.1182/blood-2013-10-525873
23. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. *J Clin Invest.* (2019) 126:2357–73. doi: 10.1172/JCI124218
24. Kanakry CG, Ganguly S, Luznik L. Situational aldehyde dehydrogenase expression by regulatory T cells may explain the contextual duality of cyclophosphamide as both a pro-inflammatory and tolerogenic agent. *Oncoimmunology.* (2015) 4:e974393. doi: 10.4161/2162402X.2014.974393
25. Berenbaum MC, Brown IN. Prolongation of homograft survival in mice with single doses of cyclophosphamide. *Nature.* (1963) 200:84. doi: 10.1038/200084a0
26. Brody GL, Jones JW, Haines RF. Influence of cyclophosphamide on homograft rejection. *JAMA.* (1965) 191:297–300. doi: 10.1001/jama.191.4.297
27. Dietrich FM, Dukor P. The immune response to heterologous red cells in mice. 3. Cyclophosphamide-induced tolerance to multispecies rd cells. *Pathol Microbiol.* (1967) 30:909–17. doi: 10.1159/000161757
28. Aisenberg AC. Studies on cyclophosphamide-induced tolerance to sheep erythrocytes. *J Exp Med.* (1967) 125:833–45. doi: 10.1084/jem.125.5.833
29. Aisenberg AC, Davis C. The thymus and recovery from cyclophosphamide-induced tolerance to sheep erythrocytes. *J Exp Med.* (1968) 128:35–46. doi: 10.1084/jem.128.1.35
30. Aisenberg AC, Wilkes B. Immunological tolerance induced by cyclophosphamide assayed by plaque spleen cell method. *Nature.* (1967) 213:498–9. doi: 10.1038/213498a0
31. Nirmul G, Severin C, Taub RN. Cyclophosphamide-induced immunologic tolerance to skin homografts. *Surg Forum.* (1971) 22:287–8.
32. Van Winkle MG. Cyclophosphamide-induced graft-versus-host immunotolerance. *J Reticuloendothel Soc.* (1971) 10:403–17.
33. Owens AH Jr, Santos GW. The effect of cytotoxic drugs on graft-versus-host disease in mice. *Transplantation.* (1971) 11:378–82. doi: 10.1097/00007890-197104000-00004
34. Nirmul G, Severin C, Taub RN. Mechanisms and kinetics of cyclophosphamide-induced specific tolerance to skin allografts in mice. *Transplant Proc.* (1973) 5:675–8.
35. Santos GW, Owens AH. Production of graft-versus-host disease in the rat and its treatment with cytotoxic agents. *Nature.* (1966) 210:139–40. doi: 10.1038/210139a0
36. Shin T, Mayumi H, Himeno K, Sanui H, Nomoto K. Drug-induced tolerance to allografts in mice. I. Difference between tumor and skin grafts. *Transplantation.* (1984) 37:580–4. doi: 10.1097/00007890-198406000-00011
37. Mayumi H, Shin T, Himeno K, Nomoto K. Drug-induced tolerance to allografts in mice II. Tolerance to tumor allografts of large doses associated with rejection of skin allografts and tumor allografts of small doses. *Immunobiology.* (1985) 169:147–61. doi: 10.1016/S0171-2985(85)80029-2
38. Mayumi H, Himeno K, Shin T, Nomoto K. Drug-induced tolerance to allografts in mice. IV. Mechanisms and kinetics of cyclophosphamide-induced tolerance. *Transplantation.* (1985) 39:209–15. doi: 10.1097/00007890-198502000-00019
39. Mayumi H, Himeno K, Shin T, Nomoto K. Drug-induced tolerance to allografts in mice. VI. Tolerance induction in H-2-haplotype-identical strain combinations in mice. *Transplantation.* (1985) 40:188–94. doi: 10.1097/00007890-198508000-00016
40. Mayumi H, Tokuda N, Fan JL, Himeno K, Nomoto K. Drug-induced tolerance to allografts in mice. XIII. Tolerance to the H-Y antigen. *Transplant Proc.* (1987) 19:2975–7.
41. Mayumi H, Kayashima K, Shin T, Nomoto K. Drug-induced tolerance to allografts in mice. V. Prolongation of skin graft survival in tolerant mice with combined immunosuppressive treatments. *Transplantation.* (1985) 39:335–7. doi: 10.1097/00007890-198503000-00032
42. Mayumi H, Himeno K, Tokuda N, Nomoto K. Drug-induced tolerance to allografts in mice. VIII. Effects of thymectomy and/or splenectomy on tolerance induction in an H-2-haplotype-identical strain combination. *Transplantation.* (1985) 40:438–41.
43. Mayumi H, Himeno K, Tokuda N, Nomoto K. Drug-induced tolerance to allografts in mice. VII. Optimal protocol and mechanism of cyclophosphamide-induced tolerance in an H-2 haplotype-identical strain combination. *Transplant Proc.* (1986) 18:363–9.
44. Mayumi H, Good RA. The necessity of both allogeneic antigens and stem cells for cyclophosphamide-induced skin allograft tolerance in mice. *Immunobiology.* (1989) 178:287–304. doi: 10.1016/S0171-2985(89)80053-1
45. Mayumi H, Himeno K, Tanaka K, Tokuda N, Fan JL, Nomoto K. Drug-induced tolerance to allografts in mice. XII. The relationships between tolerance, chimerism, and graft-versus-host disease. *Transplantation.* (1987) 44:286–90. doi: 10.1097/00007890-198708000-00021
46. Mayumi H, Good RA. Dependency of cyclophosphamide-induced skin allograft tolerance on age of adult recipient mice. *Transplantation.* (1988) 46:451–3. doi: 10.1097/00007890-198809000-00024
47. Mayumi H, Good RA. Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. *J Exp Med.* (1989) 169:213–38. doi: 10.1084/jem.169.1.213

48. Mayumi H, Good RA. Induction of tolerance across major barriers using a two-step method with genetic analysis of tolerance induction. *Immunobiology.* (1989) 179:86–108. doi: 10.1016/S0171-2985(89)80009-9
49. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Nomoto K. Sequential mechanisms of cyclophosphamide-induced skin allograft tolerance including the intrathymic clonal deletion followed by late breakdown of the clonal deletion. *J Immunol.* (1990) 145:1303–10.
50. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nomoto K. Intrathymic clonal deletion of V beta 6+ T cells in cyclophosphamide-induced tolerance to H-2-compatible, Mls-disparate antigens. *J Exp Med.* (1990) 171:97–113. doi: 10.1084/jem.171.1.97
51. Hodes RJ, Abe R. Mouse endogenous superantigens: Ms and Mls-like determinants encoded by mouse retroviruses. *Curr Protoc Immunol.* (2001) 17: A.1F.1–5. doi: 10.1002/0471142735.ima01fs17
52. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Nomoto K. The requirement of intrathymic mixed chimerism and clonal deletion for a long-lasting skin allograft tolerance in cyclophosphamide-induced tolerance. *Eur J Immunol.* (1990) 20:2005–13. doi: 10.1002/eji.1830200919
53. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Maeda T, et al. Specific destruction of host-reactive mature T cells of donor origin prevents graft-versus-host disease in cyclophosphamide-induced tolerant mice. *J Immunol.* (1991) 146:1402–9.
54. Tomita Y, Mayumi H, Eto M, Nomoto K. Importance of suppressor T cells in cyclophosphamide-induced tolerance to the non-H-2-encoded alloantigens. Is mixed chimerism really required in maintaining a skin allograft tolerance? *J Immunol.* (1990) 144:463–73.
55. Kong YY, Eto M, Omoto K, Umesue M, Hashimoto A, Nomoto K. Regulatory T cells in maintenance and reversal of peripheral tolerance *in vivo*. *J Immunol.* (1996) 157:5284–9.
56. Tomita Y, Nishimura Y, Harada N, Eto M, Ayukawa K, Yoshikai Y, et al. Evidence for involvement of clonal anergy in MHC class I and class II disparate skin allograft tolerance after the termination of intrathymic clonal deletion. *J Immunol.* (1990) 145:4026–36.
57. Colson YL, Li H, Boggs SS, Patrene KD, Johnson PC, Ildstad ST. Durable mixed allogeneic chimerism and tolerance by a non-lethal radiation-based cytoreductive approach. *J Immunol.* (1996) 157:2820–9.
58. Colson YL, Wren SM, Schuchert MJ, Patrene KD, Johnson PC, Boggs SS, et al. A non-lethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *J Immunol.* (1995) 155:4179–88.
59. Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after non-myeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood.* (2001) 98:3456–64. doi: 10.1182/blood.V98.12.3456
60. Luznik L, Engstrom LW, Iannone R, Fuchs EJ. Posttransplantation cyclophosphamide facilitates engraftment of major histocompatibility complex-identical allogeneic marrow in mice conditioned with low-dose total body irradiation. *Biol Blood Marrow Transplant.* (2002) 8:131–8. doi: 10.1053/bbmt.2002.v8.pm11939602
61. Leventhal J, Abecassis M, Miller J, Gallon L, Ravindra K, Tollerud DJ, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med.* (2012) 4:124ra28. doi: 10.1126/scitranslmed.3003509
62. Leventhal J, Abecassis M, Miller J, Gallon L, Tollerud D, Elliott MJ, et al. Tolerance induction in HLA disparate living donor kidney transplantation by donor stem cell infusion: durable chimerism predicts outcome. *Transplantation.* (2013) 95:169–76. doi: 10.1097/TP.0b013e3182782fc1
63. Mayumi H, Himeno K, Tokuda N, Fan JL, Nomoto K. Drug-induced tolerance to allografts in mice. X. Augmentation of split tolerance in murine combinations disparate at both H-2 and non-H-2 antigens by the use of spleen cells from donors preimmunized with recipient antigens. *Immunobiology.* (1987) 174:274–91. doi: 10.1016/S0171-2985(87)80003-7
64. O'Donnell P, Raj K, Pagliuca A. High fever occurring 4 to 5 days post-transplant of haploidentical bone marrow or peripheral blood stem cells after reduced-intensity conditioning associated with the use of post-transplant cyclophosphamide as prophylaxis for graft-versus-host disease. *Biol Blood Marrow Transplant.* (2015) 21:197–8. doi: 10.1016/j.bbmt.2014.10.008
65. Solomon SR, Sizemore CA, Sanacore M, Zhang X, Brown S, Holland HK, et al. Haploidentical transplantation using T cell replete peripheral blood stem cells and myeloablative conditioning in patients with high-risk hematologic malignancies who lack conventional donors is well-tolerated and produces excellent relapse-free survival: results of a prospective phase II trial. *Biol Blood Marrow Transplant.* (2012) 18:1859–66. doi: 10.1016/j.bbmt.2012.06.019
66. Powles RL, Morgenstern GR, Kay HE, McElwain TJ, Clink HM, Dady PJ, et al. Mismatched family donors for bone-marrow transplantation as treatment for acute leukaemia. *Lancet.* (1983) 1:612–5. doi: 10.1016/S0140-6736(83)91793-2
67. Mayumi H, Himeno K, Tanaka K, Tokuda N, Fan JL, Nomoto K. Drug-induced tolerance to allografts in mice. IX. Establishment of complete chimerism by allogeneic spleen cell transplantation from donors made tolerant to H-2-identical recipients. *Transplantation.* (1986) 42:417–22. doi: 10.1097/00007890-198610000-00018
68. Luznik L, Fuchs EJ. High-dose, post-transplantation cyclophosphamide to promote graft-host tolerance after allogeneic hematopoietic stem cell transplantation. *Immunol Res.* (2010) 47:65–77. doi: 10.1007/s12026-009-8139-0
69. McCurdy SR, Kanakry CG, Tsai HL, Kasamon YL, Showel MM, Bolanos-Meade J, et al. Grade II acute graft-versus-host disease and higher nucleated cell graft dose improve progression-free survival after HLA-haploidentical transplant with post-transplant cyclophosphamide. *Biol Blood Marrow Transplant.* (2018) 24:343–52. doi: 10.1016/j.bbmt.2017.10.023
70. Mielcarek M, Furlong T, O'Donnell PV, Storer BE, McCune JS, Storb R, et al. Posttransplantation cyclophosphamide for prevention of graft-versus-host disease after HLA-matched mobilized blood cell transplantation. *Blood.* (2016) 127:1502–8. doi: 10.1182/blood-2015-10-672071
71. Kasamon YL, Bolanos-Meade J, Prince GT, Tsai HL, McCurdy SR, Kanakry JA, et al. Outcomes of non-myeloablative HLA-haploidentical blood or marrow transplantation with high-dose post-transplantation cyclophosphamide in older adults. *J Clin Oncol.* (2015) 33:3152–61. doi: 10.1200/JCO.2014.60.4777
72. Nakamae H, Koh H, Katayama T, Nishimoto M, Hayashi Y, Nakashima Y, et al. HLA haploidentical peripheral blood stem cell transplantation using reduced dose of posttransplantation cyclophosphamide for poor-prognosis or refractory leukemia and myelodysplastic syndrome. *Exp Hematol.* (2015) 43:921–9 e1. doi: 10.1016/j.exphem.2015.07.006
73. Wang Y, Chang YJ, Chen L, Xu LP, Bian ZL, Zhang XH, et al. Low-dose post-transplant cyclophosphamide can mitigate GVHD and enhance the G-CSF/ATG induced GVHD protective activity and improve haploidentical transplant outcomes. *Oncoimmunology.* (2017) 6:e1356152. doi: 10.1080/2162402X.2017.1356152
74. Nomoto K, Eto M, Yanaga K, Nishimura Y, Maeda T, Nomoto K. Interference with cyclophosphamide-induced skin allograft tolerance by cyclosporin A. *J Immunol.* (1992) 149:2668–74.
75. Raiola AM, Dominietto A, Ghiso A, Di Grazia C, Lamparelli T, Gualandi F, et al. Unmanipulated haploidentical bone marrow transplantation and posttransplantation cyclophosphamide for hematologic malignancies after myeloablative conditioning. *Biol Blood Marrow Transplant.* (2013) 19:117–22. doi: 10.1016/j.bbmt.2012.08.014
76. Chiusolo P, Bug G, Olivieri A, Brune M, Mordini N, Alessandrino PE, et al. A modified post-transplant cyclophosphamide regimen, for unmanipulated haploidentical marrow transplantation, in acute myeloid leukemia: a multicenter study. *Biol Blood Marrow Transplant.* (2018) 24:1243–9. doi: 10.1016/j.bbmt.2018.01.031
77. Roberto A, Castagna L, Zanon V, Bramanti S, Crocchiolo R, McLaren JE, et al. Role of naive-derived T memory stem cells in T-cell reconstitution following allogeneic transplantation. *Blood.* (2015) 125:2855–64. doi: 10.1182/blood-2014-11-608406
78. Cieri N, Oliveira G, Greco R, Forcato M, Taccioli C, Cianciotti B, et al. Generation of human memory stem T cells after haploidentical T-replete hematopoietic stem cell transplantation. *Blood.* (2015) 125:2865–74. doi: 10.1182/blood-2014-11-608539
79. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Kanakry CG. Optimized timing of post-transplantation cyclophosphamide in MHC-haploidentical murine hematopoietic cell transplantation. *Biol Blood Marrow Transpl.* (2019). doi: 10.1016/j.bbmt.2019.09.030. [Epub ahead of print].

80. Kanakry CG, Coffey DG, Towlerton AM, Vulic A, Storer BE, Chou J, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. *JCI Insight.* (2016) 1:e86252. doi: 10.1172/jci.insight.86252
81. Nguyen VH, Zeiser R, Dasilva DL, Chang DS, Beilhack A, Contag CH, et al. *In vivo* dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood.* (2007) 109:2649–56. doi: 10.1182/blood-2006-08-044529
82. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood.* (2011) 117:3921–8. doi: 10.1182/blood-2010-10-311894
83. Brodsky RA, Sensenbrenner LL, Smith BD, Dorr D, Seaman PJ, Lee SM, et al. Durable treatment-free remission after high-dose cyclophosphamide therapy for previously untreated severe aplastic anemia. *Ann Intern Med.* (2001) 135:477–83. doi: 10.7326/0003-4819-135-7-200110020-00006
84. Cieri N, Greco R, Crucitti L, Morelli M, Giglio F, Levati G, et al. Post-transplantation cyclophosphamide and sirolimus after haploidentical hematopoietic stem cell transplantation using a treosulfan-based myeloablative conditioning and peripheral blood stem cells. *Biol Blood Marrow Transplant.* (2015) 21:1506–14. doi: 10.1016/j.bbmt.2015.04.025
85. Meyer EH, Hsu AR, Liliental J, Lohr A, Florek M, Zehnder JL, et al. A distinct evolution of the T-cell repertoire categorizes treatment refractory gastrointestinal acute graft-versus-host disease. *Blood.* (2013) 121:4955–62. doi: 10.1182/blood-2013-03-489757
86. Russo A, Oliveira G, Berglund S, Greco R, Gambacorta V, Cieri N, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood.* (2018) 131:247–62. doi: 10.1182/blood-2017-05-780668
87. Al-Homsi AS, Lai Z, Roy TS, Al-Malki MM, Kouttab N, Junghans RP. Post-transplant cyclophosphamide and bortezomib inhibit dendritic cell maturation and function and alter their IkappaB and NFkappaB. *Transpl Immunol.* (2014) 30:40–5. doi: 10.1016/j.trim.2013.11.003
88. Dodd OJ, Ganguly S, Vulic A, Panoskaltsis-Mortari A, McDyer JF, Luznik L. Induction of major histocompatibility complex-mismatched mouse lung allograft acceptance with combined donor bone marrow: lung transplant using a 12-hour nonmyeloablative conditioning regimen. *Transplantation.* (2016) 100:e140–e6. doi: 10.1097/TP.0000000000001480
89. Leventhal JR, Elliott MJ, Yolcu ES, Bozulic LD, Tollerud DJ, Mathew JM, et al. Immune reconstitution/immunocompetence in recipients of kidney plus hematopoietic stem/facilitating cell transplants. *Transplantation.* (2015) 99:288–98. doi: 10.1097/TP.0000000000000605
90. Chen YB, Elias N, Heher E, McCune JS, Collier K, Li S, et al. Haploidentical hematopoietic cell and kidney transplantation for hematological malignancies and end-stage renal failure. *Blood.* (2019) 134:211–5. doi: 10.1182/blood.2019000775
91. Morris H, DeWolf S, Robins H, Sprangers B, LoCascio SA, Shonts BA, et al. Tracking donor-reactive T cells: evidence for clonal deletion in tolerant kidney transplant patients. *Sci Transl Med.* (2015) 7:272ra10. doi: 10.1126/scitranslmed.3010760
92. McCurdy SR, Kanakry JA, Showel MM, Tsai HL, Bolanos-Meade J, Rosner GL, et al. Risk-stratified outcomes of non-myeloablative HLA-haploidentical BMT with high-dose posttransplantation cyclophosphamide. *Blood.* (2015) 125:3024–31. doi: 10.1182/blood-2015-01-623991
93. Kanakry CG, Luznik L. Teaching a young dog new tricks: modifications to the post-transplantation cyclophosphamide haploidentical transplantation platform. *Biol Blood Marrow Transplant.* (2018) 24:1108–10. doi: 10.1016/j.bbmt.2018.04.004

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$CD4^+FOXP3^+$ Regulatory T Cell Therapies in HLA Haploidentical Hematopoietic Transplantation

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Since their discovery $CD4^+FOXP3^+$ regulatory T cells (Tregs) represented a promising tool to induce tolerance in allogeneic hematopoietic cell transplantation. Preclinical models proved that adoptive transfer of Tregs or the use of compounds that can favor their function *in vivo* are effective for prevention and treatment of graft-vs.-host disease (GvHD). Following these findings, Treg-based therapies have been employed in clinical trials. Adoptive immunotherapy with Tregs effectively prevents GvHD induced by alloreactive T cells in the setting of one HLA haplotype mismatched hematopoietic transplantation. The absence of post transplant pharmacologic immunosuppression unleashes T-cell mediated graft-vs.-tumor (GvT) effect, which results in an unprecedented, almost complete control of leukemia relapse in this setting. In the present review, we will report preclinical studies and clinical trials that demonstrate Treg ability to promote donor engraftment, protect from GvHD and improve GvT effect. We will also discuss new strategies to further enhance *in vivo* efficacy of Treg-based therapies.

Keywords: regulatory T cells, allogeneic hematopoietic transplantation, tolerance, engraftment, graft-vs.-host-disease, graft-vs.-tumor effect

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INTRODUCTION

$CD4^+FOXP3^+$ regulatory T cells (Tregs) are capable of suppressing the function of conventional $CD4^+$ and $CD8^+$ T cells (Tcons), B cells, NK cells and antigen presenting cells (APCs). They maintain tolerance to self and prevent autoimmune diseases, control excessive immune responses to allergens and pathogens, help maintain a balance with commensal microbial flora and the maternal tolerance to fetus. They have been shown to infiltrate tumors and suppress anti-tumor immunity (1–6). Recent studies have uncovered a role for bone marrow mouse Tregs in the maintenance of the hematopoietic stem cell (HSC) niche and in B cell lymphopoiesis (7, 8). Tregs develop in the thymus with a T cell receptor (TCR) repertoire that overlaps to some extent with that of Tcons (9–12). In addition, studies in mice reported that TGF-β and retinoic acid can induce differentiation of peripheral naïve $CD4^+$ T cells into Tregs in response to antigenic stimulation (13–19). FOXP3 is a transcription factor of the forkhead winged helix family and is the lineage marker for both mouse and human Tregs (20–25), although human Tcons can express low levels of FOXP3 after activation (26, 27). FOXP3 deficiency causes lymphoproliferation and multi-organ autoimmunity in scurfy mice and a lethal X-linked syndrome with immune dysregulation, polyendocrinopathy, and entheropathy in humans (28–31). Recent studies in mouse models suggest that FOXP3 expression is not required to direct thymocyte development to the Treg cell lineage, but it is essential for Treg stability and function (32–34). TCR, IL-2, and TGF-β signaling can induce *Foxp3*

transcription and are responsible for the maintenance and the function of thymic Tregs and the differentiation of Tregs in the periphery (35–38). Among others, FOXP3 represses the transcription of genes for IL-2 and other inflammatory cytokines, while it activates the transcription of *IL2RA*, *CTLA4,* and its own gene (39, 40). The α chain of the IL-2 receptor (IL-2Rα, CD25) and CTLA-4 are constitutively expressed by Tregs, while they are expressed on Tcons upon activation (1, 2, 41–44). Due to the intranuclear localization of FOXP3, CD25 is currently used to select Tregs for functional *in vitro* studies and cellular therapy, although it is not an exclusive marker. As Tregs do not produce IL-2, they depend on IL-2 produced by other immune cells, mainly Tcons (45). Tregs constitutively express the high-affinity receptor for IL-2 (IL-2Rαβγ_c), thus they can efficiently compete for IL-2 with Tcons and NK cells (46–48). TCR stimulation activates Tregs that are capable of suppressing antigen-specific responses but can also exert bystander suppression (49). Tregs act through several mechanisms, including production of inhibitory cytokines such us IL-10 and TGF-β, cell-cell contacts, and cytolysis (5, 50). The inhibition of dendritic cell maturation and function is considered a core mechanism of Treg-mediated suppression. CTLA-4 on Tregs binds CD80 and CD86 on dendritic cells and inhibits maturation of APCs and co-stimulation of Tcons (51, 52).

Efforts have been made to exploit Treg function for the treatment of autoimmune and inflammatory diseases. Recently, clinical trials are evaluating Treg-based therapies in allogeneic hematopoietic transplantation (HCT) with promising results. Allogeneic HCT is a life-saving treatment for high-risk hematologic malignancies (53). After a conditioning regimen based on radiotherapy and/or chemotherapy, a bone marrow or a stem cell graft reconstitutes hematopoiesis and immunity of donor origin in the recipient. Residual host T cells that may have survived the conditioning regimen can recognize donor alloantigens and cause rejection. Similarly, donor alloreactive T cells recognize recipient alloantigens, eliminate malignant cells [graft-vs.-tumor (GvT) effect] and can prevent relapse. However, they also attack host normal tissues (mainly skin, gut and liver), causing graft-vs.-host disease (GvHD), that is a major cause of non-relapse mortality (NRM). Pharmacological immune suppression is widely used to prevent GvHD, but it also reduces the GvT effect. Separation of the GvT effect from GvHD is the main goal of the translational research in the field. In this setting, Tregs contribute to induction and maintenance of tolerance to alloantigens, facilitating engraftment and preventing the development of GvHD (54–56).

INDUCTION OF TOLERANCE BY TREGS IN PRECLINICAL MODELS OF ALLOGENEIC HCT

Mouse models of allogeneic HCT have provided evidence that Tregs suppress T cell alloreactions and can promote engraftment and help control GvHD. The role of Tregs in GvHD has been mostly investigated in MHC mismatched bone marrow transplantation. Pioneering studies demonstrated that $CD4^+CD25^+$ T-cell depletion from the bone marrow graft exacerbated GvHD. When additional $CD4^+CD25^+$ T cells, either freshly isolated or *ex-vivo* expanded, were infused, GvHD onset was delayed and even prevented to various degrees (57–60). One study showed that Tregs also protected from GvHD in a minor histocompatibility antigen-disparate, MHC matched setting (60). In a mouse model of fully mismatched T-cell depleted bone marrow transplantation, infusion of donor Tcons killed all the mice within 30 days of acute GvHD. When donor $CD4^+CD25^+$ Tregs were co-infused at a 1:1 ratio with Tcons more than 70% of mice were protected from lethal acute GvHD. Co-infusion of Tregs reduced the number of Tcons that could be recovered in lymph nodes and GvHD target tissues such as skin and gut, thus limiting Tcon expansion (61). Importantly, when mice were co-injected with a leukemia or lymphoma cell line, transfer of Tregs did not inhibit Tcon-mediated GvT effect (60–62). Moreover, Treg transfer preserved thymic and lymph node architecture and even accelerated donor lymphoid reconstitution to such an extent that mice survived lethal mouse Cytomegalovirus (CMV) infection (63). Treg homing to lymph nodes and target tissue is an important variable in GvHD prevention. In mouse models of GvHD, bioluminescence analyses showed that Tregs localized to peripheral lymph nodes and spleen in the first 24–48 h with a peak on day 4 after infusion, then they migrated to peripheral tissues. When Tregs were infused 2 days before Tcons, an unfavorable 1:10 ratio with Tcons still protected from GvHD at some extent (64). Moreover, when Tregs were eliminated *in vivo* 2 days after their transfer and even before Tcon injection, mice survived to GvHD (65). Finally, $CD62L^-$ Tregs do not migrate to lymph nodes and are not capable of controlling GvHD (66, 67). Thus, Tregs migrate to lymph nodes and suppress Tcon proliferation early after transplant. However, Tregs could also suppress alloreactive Tcons in GvHD target tissues. One study showed that CCR5-deficient Tregs had reduced migration to mesenteric lymph nodes, liver, lung, and spleen and were less effective in preventing GvHD (68). In another study, CXCR3-transfected Tregs migrated better to liver, lung, and intestine and better controlled GvHD (69). Adoptively transferred third party Tregs also conferred protection from GVHD in mouse models of allogeneic transplantation, although they were less effective than donor Tregs. Third party Tregs survived for a shorter period, probably because they were rejected by donor Tcons. Thus, suppression of alloreactive T cells can also operate through MHC-independent mechanisms (65). Moreover, both radiation-resistant host Tregs and donor Tregs reduced the severity of chronic GvHD in a mouse model of bone marrow transplantation with a minor histocompatibility antigen mismatch (70). The capability of human donor Tregs to prevent and ameliorate GvHD caused by co-infused Tcons has been demonstrated in xenogeneic mouse models (71–73), and one of these studies also showed that the GvT effect was unaffected (73). Similar results were obtained with the infusion of third party human Tregs derived from umbilical cord blood and expanded before infusion (74). The same group also showed that fucosylation of expanded third party Tregs improved prevention of GvHD. In fact, the addition of a fucose formed the moiety found on P-selectin ligand on Treg cell surface, enhancing their

persistence *in vivo* as a result of improved homing to the sites of inflammation (75).

Other studies focused more specifically on the role of Tregs in the engraftment of T-cell depleted bone marrow allografts. Joffre et al. demonstrated that when host Tregs were activated *in vitro* with donor allogeneic APCs, they inhibited host $CD4^+$ and $CD8^+$ T-cell mediated rejection of donor bone marrow graft, but not of a third-party bone marrow graft (76, 77). Another study showed that either donor or host $CD62L^{hi}$, but not $CD62L^{lo}$, *ex-vivo* activated Tregs inhibited rejection of MHC-mismatched bone marrow in sublethally irradiated mice (66). In a similar model, donor Tregs promoted engraftment without being activated *ex-vivo* (78). Steiner et al. showed that third-party Tregs, either naïve or *ex-vivo* expanded, enhanced engraftment of bone marrow allografts (79). Adoptive transfer of host-type Tregs can also induce mixed chimerism and tolerance to a fully mismatched bone marrow graft after a conditioning with short-course costimulation blockade (with CTLA-4 Ig and anti-CD40L antibody) and rapamycin, in the absence of cytoreductive treatment (80). Rapamycin is used because it limits activation and expansion of Tcons while promoting the expansion of Tregs (81). The ability of Tregs to promote engraftment is consistent with their role in the maintenance of the HSC niche, where they protect HSCs from autologous and allogeneic immune attack (7, 8). Tregs are also critical for tolerance induction to allogeneic HCT after reduced intensity conditioning regimens with total lymphoid irradiation and anti-thymocyte globulin (TLI/ATG). These regimens kill host-type Tcons while partially sparing Tregs, which increase donor HSC engraftment, cell cycling and differentiation (82, 83). These results support the use of TLI/ATG to establish mixed chimerism after allogeneic HCT in patients with hematologic malignancies (84).

TREG-BASED THERAPIES IN ONE HLA HAPLOTYPE MISMATCHED TRANSPLANTATION

In one HLA haplotype mismatched (haploidentical) hematopoietic transplantation, the high degree of HLA mismatch triggers strong host-vs.-graft and graft-vs.-host alloresponses. In the early 1990s, the combination of a myeloablative and immunosuppressive conditioning regimen with the infusion of a "mega-dose" of T-cell-depleted HSCs made haploidentical transplantation feasible and effective without the need for any post-transplant GvHD prophylaxis. The major limitation of this approach was delayed post-transplant immune recovery, which resulted in ∼40% NRM, mainly due to infections (85–87). More recently, protocols of unmanipulated (T-cell replete) haploidentical transplantation have been developed. They are based on new strategies to control T-cell alloreactivity that also rely on Treg-induced tolerance (87, 88). A widespread and effective approach is the administration of high-dose cyclophosphamide following graft infusion (PTCy), which inhibits alloresponses while sparing donor HSCs (88–90). Donor Tregs are resistant to PTCy-induced cytotoxicity upon allogeneic HCT, because they express high levels of the enzyme aldehyde dehydrogenase, which is essential for *in vivo* detoxification of cyclophosphamide (91). Treg-mediated suppression plays an essential role in the prevention of GvHD in mouse models of allogeneic HCT with PTCy (91, 92). A recent study showed that PTCy induced functional impairment rather than elimination of alloreactive T cells and confirmed a role for Tregs in GvHD mouse models (93). Moreover, recovery of mature and functional natural killer (NK) cells is also impaired in patients undergoing haploidentical transplantation with PTCy (94, 95). Such effects could impair T cell- and NK cell-mediated leukemia killing after transplant. Importantly, clinical data suggest survival after haploidentical transplantation with PTCy is similar to that of patients undergoing HLA-matched sibling or unrelated HCT (96–102). Another trial used a rapamycin-based GvHD prophylaxis in order to promote *in vivo* expansion of Tregs and allow the infusion of unmanipulated haploidentical grafts (103).

In the setting of T-cell depleted haploidentical transplantation without post-transplant immunosuppression, the Perugia Bone Marrow Transplant Program is exploiting adoptive immunotherapy with freshly isolated donor Tcons and Tregs, in order to promote immune reconstitution while preventing GvHD (73, 87, 104, 105). $CD4^+CD25^+$ Tregs are isolated by a two-step immunomagnetic selection consisting of a negative CD19/CD8 selection, followed by a positive CD25 selection. The purity of $CD4^+FOXP3^+$ Tregs is around 70–80%. 2×10^6 Tregs/kg are infused 4 days before 1×10^6 Tcons/kg and about 10×10^6 $CD34^+$ cells/Kg. No pharmacological GvHD prophylaxis is given. The first study reported results in 28 patients with hematologic malignancies (24 in any complete remission and 4 in relapse at transplant). They received a conditioning regimen with TBI, thiotepa, fludarabine, and cyclophosphamide. Twenty-six patients engrafted. Treg/Tcon adoptive immunotherapy was associated with rapid reconstitution of B cells and of T cells with a wide repertoire. Compared with standard T-cell depleted haploidentical transplantation, reconstitution of pathogen-specific $CD4^+$ and $CD8^+$ T cells, and of mature NK cells was faster. The incidence of CMV reactivation was markedly reduced, with no CMV-related deaths. Two patients developed ≥grade 2 acute GvHD and no patient developed chronic GvHD. One patient (in chemoresistant relapse at transplant) relapsed. Thirteen patients died of NRM, 4 of them because of extra-hematological toxicity (104). The second study extended the analysis to 43 patients with high-risk leukemia in any remission, including 24 reported before. In order to reduce NRM, a lower dose of cyclophosphamide or anti-T cell antibodies in the place of cyclophosphamide were used in the conditioning regimen. Forty-one patients engrafted. NRM was 40% and fell to 21% in patients who had received anti-T cell antibodies as a part of the conditioning. Incidence of ≥grade 2 acute GvHD was 15% and only one patient developed chronic GvHD. Incidence of leukemia relapse was 5% and it was significantly reduced compared to the standard protocol of T-cell depleted haploidentical transplantation (73). Finally, in an updated analysis of a total of 60 patients with acute leukemia in any remission at transplant, incidence of acute grade II-IV GvHD and

chronic GvHD were 15 and 3%, respectively. Five-year incidence of NRM was 35%. Five-year relapse incidence was as low as 12%, confirming preclinical data that Treg adoptive transfer does not impair T-cell immune reconstitution and the GvT effect (87). More recently, this strategy of adoptive immunotherapy with Treg/Tcon in haploidentical transplantation has been combined with a low-toxicity conditioning regimen based on total marrow and lymphoid irradiation (TMLI). TMLI provides high-intensity irradiation to bone marrow, spleen, and major lymph node chains, while sparing other tissues (106). This transplant protocol has been tested in acute myeloid leukemia patients who were aged or unfit to receive total body irradiation. Preliminary data indicate this combination provides promising results in terms of control of leukemia relapse and chronic GvHD/leukemia-free survival (107). Despite acute GvHD still occurs in a relevant fraction of patients, Treg/Tcon adoptive immunotherapy strikingly improves outcomes of T-cell depleted haploidentical transplantation thanks to a better chronic GvHD/leukemia-free survival.

TREG-BASED THERAPIES IN HLA-MATCHED AND UMBILICAL CORD BLOOD TRANSPLANTATION

Since the number of freshly isolated Tregs to be used for cellular therapy is limited (5–10% of $CD4^+$ T cells in peripheral blood), several protocols of expansion under good manufacturing practice have been developed and tested in clinical trials (108). Such expansion protocols preserve $FOXP3^+$ Treg purity and suppressive function *in vitro*. Tregs can be isolated from peripheral blood or umbilical cord blood by immunomagnetic selection as described above, or by flow cytometric cell sorting. Tregs are usually incubated with anti-CD3/CD28 beads and high-dose IL-2 with or without rapamycin for around 2–3 weeks (108–112). Another option is the use of a modified K562 cell line, which expresses the Fc receptor CD64 to cross-link an anti-CD3 antibody, and CD86 to provide co-stimulation in the presence of IL-2 (113–115). Brunstein and colleagues used this approach to prevent GvHD after double-umbilical cord blood transplantation. Tregs were isolated by CD25 immunomagnetic selection from a third party cord blood unit that was 4–6/6 HLA matched with the patient, and expanded *in vitro* (114). In the last published clinical study, 3–100 $\times$ 10^6 Tregs/kg were infused in 11 patients with various hematologic malignancies, who also received post-transplant immune suppression. Expanded Tregs were detectable in patients for a maximum of 14 days after the infusion. The incidence of grade II-IV acute GvHD at 100 days was 9% compared with 45% in contemporary controls with the same transplant protocol without Treg infusion. Chronic GvHD was 0% compared with 14% in controls. Incidence of infections, NRM and relapse, and disease-free survival were similar in patients infused with Tregs and in controls (114). Such studies suggest third party Tregs can be an alternative to donor-derived Tregs. However, donor and third party Tregs have never been compared in clinical studies for their efficacy in GvHD prevention. The infusion of donor expanded Tregs has been also tested for the treatment of acute and chronic GvHD in small series of patients after HLA-matched transplantation, with a clinical response in some patients (109, 111).

A recent phase I/II study investigated the infusion of fresh donor Tregs and Tcons in the setting HLA-matched transplantation in 12 patients with various hematological malignancies (116). Tregs were purified by $CD25^+$ immunomagnetic selection, followed by flow cytometric cell sorting of $CD4^+CD127^{lo}CD25^+$ cells (as Treg cells do not express the IL-7 receptor α subunit CD127 or express it at low intensity). Purity of $CD4^+FOXP3^+$ Tregs was over 90%. The first cohort of 5 patients received frozen Tregs but showed signs of GvHD, consistent with the reduced functionality of Tregs after cryopreservation and thawing (117). The other 7 patients received freshly isolated Tregs in combination with low-dose single-agent GvHD prophylaxis. No acute or chronic GvHD was observed (116).

TREATMENT WITH LOW-DOSE IL-2 IN ALLOGENEIC HCT

A promising alternative to Treg adoptive transfer is low-dose IL-2 therapy, that has been shown to selectively activate and expand Tregs in several autoimmune and inflammatory settings, due to the constitutive expression of CD25 and factors related to IL-2 receptor signaling (118, 119). The group from Dana-Faber Cancer Institute and Harvard University investigated *in vivo* stimulation of Tregs with low-dose IL-2 for the treatment of steroid-refractory chronic GvHD after allogeneic HCT. A phase I study established that the maximum tolerated dose of IL-2 was 1 $\times$ 10^6 IU/m^2/day. During the 8 weeks of treatment, Treg counts and the Treg:Tcon ratio rose and 52% of patients had a clinical response (120). In a phase II study, 35 patients with steroid-refractory chronic GvHD were treated for 12 weeks. Two patients withdrew, 5 patients required dose reduction, and 61% of evaluable patients had a partial response to treatment. After a 4-week hiatus, low-dose IL-2 therapy could be extended for 2 years in patients with partial response or stable disease (121). The same group showed that low-dose IL-2 induced phosphorylation of signal transducer and activator of transcription 5 (STAT5) in Tregs but not in Tcons and preferentially expanded Tregs *in vitro*. Moreover, patients with severe chronic GvHD had constitutive phosphorylation of STAT5 in Tcons and high levels of IL-7 and IL-15. Treatment with low-dose IL-2 was associated with increased STAT5 phosphorylation in Tregs and decreased STAT5 phosphorylation in Tcons. This resulted in enhanced Treg proliferation, thymic export and resistance to apoptosis (122). Strategies to enhance efficacy of low-dose IL-2 therapy are under investigation and include combination with infusion of purified Tregs or extracorporeal photopheresis (123, 124). Another phase II study used ultra-low dose IL-2 for GvHD prophylaxis in 16 pediatric recipients of allogeneic HCT. Treatment started at a median of 28 days after transplant and consisted of 1–2 $\times$ 10^5 IU/m^2 three times per week for 6 or 12 weeks. This treatment was safe and it was associated with expansion of Tregs *in vivo*. None of the patients

developed II-IV GvHD, while the incidence of acute GvHD was 12% in a control group of patients who did not receive IL-2 treatment (125).

PERSPECTIVES

Recent clinical trials demonstrate that Treg-based therapies can effectively promote engraftment and prevent or treat GvHD after allogeneic HCT. However, these treatments fail to protect some patients from severe GvHD. Strategies to improve purity, enhance specificity, promote activation and control localization of Tregs are needed. Several experimental strategies are under investigation to achieve this goal.

Recent studies have focused on the generation of IL-2 compounds with enhanced selectivity for Tregs (126). One strategy is the use of anti-IL-2 antibodies in complex with IL-2 that allow for selective stimulation of T cell subsets. Boyman et al. showed that one anti-mouse IL-2 antibody prevented the binding of IL-2 to IL-2R$\beta\gamma_c$, but not to CD25 (IL-2Rα). When complexed with IL-2, this antibody preferentially triggered the proliferation of Tregs (127). Subsequently, Trotta et al. reported the generation of a fully human anti-IL-2 antibody (F5111.2) that exerted the same effect on human Tregs when complexed with IL-2. Treatment with F5111.2-human IL-2 complexes was effective in preclinical models of autoimmune diseases and GvHD, and it did not affect immune response to mouse CMV (128). IL-2 can also be covalently linked to the antibody to generate a single-agent cytokine/antibody fusion that is more stable than the above immune complexes (129). Ward et al. designed a fusion protein of mouse IL-2 and CD25 that allows for a selective stimulation of Tregs *in vivo*. A treatment with this fusion protein delayed the development of diabetes in non-obese diabetic mice (130). An alternative strategy is engineering human IL-2 so that it preferentially binds the high affinity receptor IL-2R$\alpha\beta\gamma_c$ and, consequently, preferentially activates Tregs (131, 132). Moreover, these IL-2 muteins are also designed to have a longer half-life and can provide a persistent stimulation of Tregs (130–132).

Another pleiotropic cytokine, TNF-α, has been recently shown to enhance Treg function. After the conditioning regimen, tissue macrophages release TNF-α, which activates donor alloreactive T cells that cause GvHD (133, 134). In fact, anti-TNF-α therapy with infliximab and etanercept is used to treat steroid-refractory chronic GvHD. However, some patients do not respond or even worsen (134–136). Tregs express higher levels of TNF receptor (TNFR) 2 compared with Tcons, and $TNFR2^+$ Tregs are more suppressive than $TNFR2^-$ Tregs (137–140). While $TNFR1^{-/-}$ mice have defective immunity to infections and inflammatory response, $TNFR2^{-/-}$ mice are affected by exacerbated inflammation (141). Several reports suggest that TNF-α/TNFR2 signaling is required for effective mouse Treg development in the thymus and optimal function

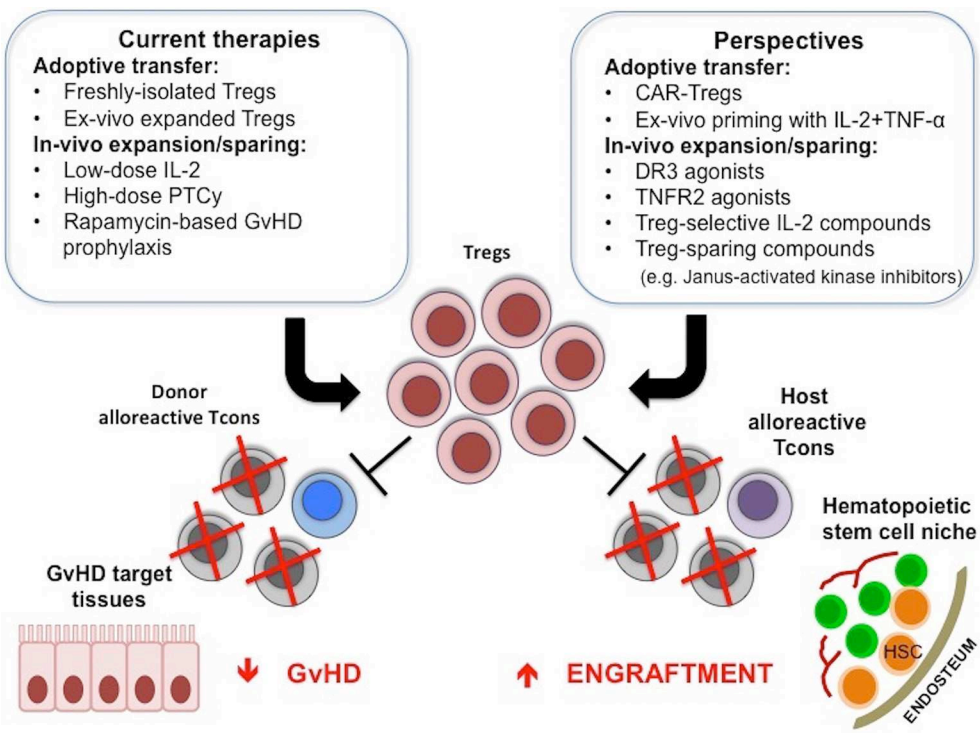

FIGURE 1 | Treg-based therapies in allogeneic HCT. Adoptive transfer of Tregs or the use of compounds that can favor their function *in vivo* are used to promote donor engraftment and protect from GvHD after HCT. New strategies to further enhance *in vivo* efficacy of Treg-based therapies are under active investigation, and include CAR-Tregs, *ex-vivo* priming with IL-2 and TNF-α, TNFR2, or DR3 agonists, Treg-selective IL-2 compounds or compounds that inhibit Tcon function while sparing Treg suppressive activity.

in vivo (142–145). In mouse models of allogeneic bone marrow transplantation, GvHD control was abrogated when mice were infused with TNFR2 deficient Tregs or with a TNFR2 blocking monoclonal antibody (143). Moreover, *ex-vivo* priming of Tregs with TNF-α enhanced the effectiveness of GvHD prevention (144). Finally, expansion of radiation resistant host Tregs with a TNFR2 agonist reduced GvHD severity and improved survival (145). Importantly, the GvT effect was unaffected by Treg stimulation in these studies. *Ex-vivo* priming or expansion of Tregs with TNF-α or a TNFR2 agonist before adoptive transfer could improve prevention and treatment of GvHD. TNFR2 agonists could also be used to expand Tregs *in vivo* (146, 147). As Tregs express higher levels of TNFR2, they could be preferentially activated. However, Tcons upregulate TNFR2 expression upon activation and become more resistant to Treg-mediated suppression (148). Thus, the effects of *in vivo* TNFR2 signaling stimulation should be carefully evaluated. Another option is treatment with agonists of the costimulatory receptor TNFR superfamily 25 (Death receptor 3, DR3), which strongly stimulate Treg proliferation while weakly affect $CD4^+$ T cell proliferation (149, 150). Treatment of donor mice with DR3 agonists reduced severity of GvHD in recipient mice of MHC-mismatched bone marrow transplantation, preserving GvT effects (151). Moreover, when recipient mice received a prophylactic treatment with a DR3 agonist, recipient-derived Treg expanded and severity of GvHD was reduced. In contrast, treatment of recipient mice after transplant favored donor Tcon alloreactions and worsened GvHD (152). In other studies, Treg expansion was induced in donors with the combination of a DR3 agonist and low-dose IL-2. The infusion of donor expanded Tregs ameliorated GvHD but did not affect GvT activity in both MHC matched and MHC mismatched bone marrow transplantation models (153–155). Recently, Copsel et al. combined this approach with the administration of inhibitors of bromodomain and extra-terminal proteins (BETi), which suppress expression of pro-inflammatory cytokines and other genes involved in T cell activation. They found that BETi EP11313 spared Tregs and that GvHD severity was reduced in mice treated with EP11313 and low numbers of donor Tregs expanded with DR3 agonist and low-dose IL-2 (156).

While current protocols of adoptive transfer use polyclonal Tregs, antigen-specific Tregs could be more effective without exerting a broad suppression of immune responses. Alloantigen-specific Tregs can be generated in the presence of allogeneic APCs or by TCR gene transfer. An alternative is the generation of chimeric antigen receptors Tregs (CAR-Tregs) (157). MacDonald et al. generated human HLA-A2-specific CAR-Tregs that prevented GvHD caused by HLA-$A2^+$ Tcons in a xenogeneic mouse model. They were more effective than Tregs expressing an irrelevant CAR (158). A similar strategy was also used to prevent rejection after xenogeneic transplantation (159). Another study used Tregs expressing a CAR that binds Fluorescein isothiocyanate-conjugated monoclonal antibodies (mAbCAR-Tregs). Using tissue-specific antibodies, mAbCAR-Tregs could be directed to different sites, where they exerted their suppressive function (160).

Finally, several compounds are under investigation for the prevention and treatment of GvHD, such as Janus-activated kinase inhibitors and others. They share the ability to preferentially inhibit Tcon function while sparing Treg suppressive activity to various degrees (161). Thus, Tregs play a key role in many of the current and newly developed strategies to induce tolerance in allogeneic HCT.

CONCLUSIONS

Treg-based therapies have provided promising clinical responses in allogeneic HCT. These treatments have proven to be safe and are not associated with the side effects that could have been anticipated, such as increased susceptibility to infections and leukemia relapse. Efforts are ongoing to improve the effectiveness of these approaches, whether they are based on adoptive transfer of freshly isolated, expanded or modified Tregs or on the induction of Treg expansion and function *in vivo* (**Figure 1**).

To these days, treatment of patients with chemoresistant leukemia is still largely ineffective. In the present review, we discussed that Treg-adoptive transfer allows for a strong Tcon-mediated GvT effect while controlling GvHD. Although it requires further optimization, we believe this strategy is close to become an effective treatment for these patients.

AUTHOR CONTRIBUTIONS

AM and SP wrote the manuscript. AV reviewed the manuscript. AP wrote and reviewed the manuscript.

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REFERENCES

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. (1995) 155:1151–64.
2. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, et al. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol*. (1998) 10:1969–80. doi: 10.1093/intimm/10.12.1969
3. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*. (2007) 8:191–7. doi: 10.1038/ni1428
4. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. (2008) 133:775–87. doi: 10.1016/j.cell.2008.05.009
5. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. $FOXP3^+$ regulatory T cells in the human immune system. *Nat Rev Immunol*. (2010) 10:490–500. doi: 10.1038/nri2785

6. Wing JB, Tanaka A, Sakaguchi S. Human FOXP3$^+$ regulatory T cell heterogeneity and function in autoimmunity and cancer. *Immunity*. (2019) 50:302–16. doi: 10.1016/j.immuni.2019.01.020
7. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, et al. *In vivo* imaging of Treg cells providing immune privilege to the haematopoietic stem- cell niche. *Nature*. (2011) 474:216–9. doi: 10.1038/nature10160
8. Pierini A, Nishikii H, Baker J, Kimura T, Kwon HS, Pan Y, et al. Foxp3$^+$ regulatory T cells maintain the bone marrow microenvironment for B cell lymphopoiesis. *Nat Commun*. (2017) 8:15068. doi: 10.1038/ncomms15068
9. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, et al. Thymus and autoimmunity: production of CD25$^+$CD4$^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol*. (1999) 162:5317–26.
10. Darrasse-Jèze G, Marodon G, Salomon BL, Catala M, Klatzmann D. Ontogeny of CD4$^+$CD25$^+$ regulatory/suppressor T cells in human fetuses. *Blood*. (2005) 105:4715–21. doi: 10.1182/blood-2004-10-4051
11. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4$^+$CD25$^+$ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. (2001) 2:301–6. doi: 10.1038/86302
12. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol*. (2006) 7:401–10. doi: 10.1038/ni1318
13. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4$^+$CD25$^-$ naive T cells to CD4$^+$CD25$^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. (2003) 198:1875–86. doi: 10.1084/jem.20030152
14. Apostolou I, von Boehmer H. *In vivo* instruction of suppressor commitment in naive T cells. *J Exp Med*. (2004) 199:1401–8. doi: 10.1084/jem.20040249
15. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*. (2005) 6:1219–27. doi: 10.1038/ni1265
16. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med*. (2007) 204:1765–74. doi: 10.1084/jem.20070719
17. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. (2007) 317:256–60. doi: 10.1126/science.1145697
18. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. (2007) 204:1775–85. doi: 10.1084/jem.20070602
19. Coombes JL, Siddiqui KR, Arancibia-Cárcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103$^+$ DCs induces Foxp3$^+$ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med*. (2007) 204:1757–64. doi: 10.1084/jem.20070590
20. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4$^+$CD25$^+$ T regulatory cells. *Nat Immunol*. (2003) 4:337–42. doi: 10.1038/ni909
21. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat Immunol*. (2003) 4:330–6. doi: 10.1038/ni904
22. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. (2003) 299:1057–61. doi: 10.1126/science.1079490
23. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, et al. Crucial role of FOXP3 in the development and function of human CD25$^+$CD4$^+$ regulatory T cells. *Int Immunol*. (2004) 16:1643–56. doi: 10.1093/intimm/dxh165
24. Roncador G, Brown PJ, Maestre L, Hue S, Martínez-Torrecuadrada JL, Ling KL, et al. Analysis of FOXP3 protein expression in human CD4$^+$CD25$^+$ regulatory T cells at the single-cell level. *Eur J Immunol*. (2005) 35:1681–91. doi: 10.1002/eji.200526189
25. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4$^+$ T cells expressing the FoxP3 transcription factor. *Immunity*. (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019
26. Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci USA*. (2006) 103:6659–64. doi: 10.1073/pnas.0509484103
27. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4$^+$ T cells. *Eur J Immunol*. (2007) 37:129–38. doi: 10.1002/eji.200636435
28. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. (2001) 27:68–73. doi: 10.1038/83784
29. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. (2001) 27:18–20. doi: 10.1038/83707
30. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. (2001) 27:20–1. doi: 10.1038/83713
31. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest*. (2000) 106:R75–81. doi: 10.1172/JCI11679
32. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. (2007) 445:771–5. doi: 10.1038/nature05543
33. Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB, et al. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol*. (2007) 8:359–68. doi: 10.1038/ni1445
34. Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. *Immunity*. (2008) 28:100–11. doi: 10.1016/j.immuni.2007.11.021
35. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. (2010) 463:808–12. doi: 10.1038/nature08750
36. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity*. (2012) 37:785–99. doi: 10.1016/j.immuni.2012.09.010
37. Feng Y, van der Veeken J, Shugay M, Putintseva EV, Osmanbeyoglu HU, Dikiy S, et al. A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. *Nature*. (2015) 528:132–6. doi: 10.1038/nature16141
38. Iizuka-Koga M, Nakatsukasa H, Ito M, Akanuma T, Lu Q, Yoshimura A. Induction and maintenance of regulatory T cells by transcription factors and epigenetic modifications. *J Autoimmun*. (2017) 83:113–21. doi: 10.1016/j.jaut.2017.07.002
39. Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci USA*. (2005) 102:5138–43. doi: 10.1073/pnas.0501675102
40. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell*. (2006) 126:375–87. doi: 10.1016/j.cell.2006.05.042
41. Read S, Malmström V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. (2000) 192:295–302. doi: 10.1084/jem.192.2.295
42. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4$^+$CD25$^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity*. (2000) 12:431–40. doi: 10.1016/S1074-7613(00)80195-8
43. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med*. (2000) 192:303–10. doi: 10.1084/jem.192.2.303

44. Paust S, Lu L, McCarty N, Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci USA*. (2004) 101:10398–403. doi: 10.1073/pnas.0403342101
45. Owen DL, Mahmud SA, Vang KB, Kelly RM, Blazar BR, Smith KA, et al. Identification of cellular sources of IL-2 needed for regulatory T cell development and homeostasis. *J Immunol*. (2018) 200:3926–33. doi: 10.4049/jimmunol.1800097
46. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. $CD4^+CD25^+Foxp3^+$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector $CD4^+$ T cells. *Nat Immunol*. (2007) 8:1353–62. doi: 10.1038/ni1536
47. Gasteiger G, Hemmers S, Firth MA, Le Floc'h A, Huse M, Sun JC, et al. IL-2-dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells. *J Exp Med*. (2013) 210:1167–78. doi: 10.1084/jem.20122462
48. Sitrin J, Ring A, Garcia KC, Benoist C, Mathis D. Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. *J Exp Med*. (2013) 210:1153–65. doi: 10.1084/jem.20122248
49. Thornton AM, Shevach EM. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J Immunol*. (2000) 164:183–90. doi: 10.4049/jimmunol.164.1.183
50. Shevach EM. Mechanisms of $foxp3^+$ T regulatory cell-mediated suppression. *Immunity*. (2009) 30:636–45. doi: 10.1016/j.immuni.2009.04.010
51. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. $Foxp3^+$ natural regulatory T cells preferentially form aggregates on dendritic cells *in vitro* and actively inhibit their maturation. *Proc Natl Acad Sci USA*. (2008) 105:10113–8. doi: 10.1073/pnas.0711106105
52. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over $Foxp3^+$ regulatory T cell function. *Science*. (2008) 322:271–5. doi: 10.1126/science.1160062
53. Appelbaum FR, Forman SJ, Negrin RS, Blume KG. *Thomas' Hematopoietic Cell Transplantation*. Oxford: Wiley-Blackwell (2009). doi: 10.1002/9781444303537
54. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol*. (2003) 3:199–210. doi: 10.1038/nri1027
55. Nguyen VH, Zeiser R, Negrin RS. Role of naturally arising regulatory T cells in hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. (2006) 12:995–1009. doi: 10.1016/j.bbmt.2006.04.009
56. Pierini A, Alvarez M, Negrin RS. NK cell and $CD4^+FoxP3^+$ regulatory T cell based therapies for hematopoietic stem cell engraftment. *Stem Cells Int*. (2016) 2016:9025835. doi: 10.1155/2016/9025835
57. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J Exp Med*. (2002) 196:401–6. doi: 10.1084/jem.20020090
58. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med*. (2002) 196:389–99. doi: 10.1084/jem.20020399
59. Taylor PA, Lees CJ, Blazar BR. The infusion of *ex vivo* activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood*. (2002) 99:3493–9. doi: 10.1182/blood.V99.10.3493
60. Jones SC, Murphy GF, Korngold R. Post-hematopoietic cell transplantation control of graft-versus-host disease by donor CD425 T cells to allow an effective graft-versus-leukemia response. *Biol Blood Marrow Transplant*. (2003) 9:243–56. doi: 10.1053/bbmt.2003.50027
61. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. $CD4^+CD25^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med*. (2003) 9:1144–50. doi: 10.1038/nm915
62. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, et al. Recipient-type specific $CD4^+CD25^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest*. (2003) 112:1688–96. doi: 10.1172/JCI17702
63. Nguyen VH, Shashidhar S, Chang DS, Ho L, Kambham N, Bachmann M, et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood*. (2008) 111:945–53. doi: 10.1182/blood-2007-07-103895
64. Nguyen VH, Zeiser R, Dasilva DL, Chang DS, Beilhack A, Contag CH, et al. *In vivo* dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood*. (2007) 109:2649–56. doi: 10.1182/blood-2006-08-044529
65. Pierini A, Colonna L, Alvarez M, Schneidawind D, Nishikii H, Baker J, et al. Donor requirements for regulatory T cell suppression of murine graft-versus-host disease. *J Immunol*. (2015) 195:347–55. doi: 10.4049/jimmunol.1402861
66. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, Lucas PJ, Gress RE, Levine BL, et al. L-Selectin(hi) but not the L-selectin(lo) $CD4^+25^+$ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood*. (2004) 104:3804–12. doi: 10.1182/blood-2004-05-1850
67. Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, Higgins JP, et al. Only the $CD62L^+$ subpopulation of $CD4^+CD25^+$ regulatory T cells protects from lethal acute GVHD. *Blood*. (2005) 105:2220–6. doi: 10.1182/blood-2004-05-2044
68. Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, Su L, et al. Critical role for CCR5 in the function of donor $CD4^+CD25^+$ regulatory T cells during acute graft-versus-host disease. *Blood*. (2005) 106:3300–7. doi: 10.1182/blood-2005-04-1632
69. Hasegawa H, Inoue A, Kohno M, Lei J, Miyazaki T, Yoshie O, et al. Therapeutic effect of CXCR3-expressing regulatory T cells on liver, lung and intestinal damages in a murine acute GVHD model. *Gene Ther*. (2008) 15:171–82. doi: 10.1038/sj.gt.3303051
70. Anderson BE, McNiff JM, Matte C, Athanasiadis I, Shlomchik WD, Shlomchik MJ. Recipient $CD4^+$ T cells that survive irradiation regulate chronic graft-versus-host disease. *Blood*. (2004) 104:1565–73. doi: 10.1182/blood-2004-01-0328
71. Mutis T, van Rijn RS, Simonetti ER, Aarts-Riemens T, Emmelot ME, van Bloois L, et al. Human regulatory T cells control xenogeneic graft-versus-host disease induced by autologous T cells in $RAG2^{-/-}gammac^{-/-}$ immunodeficient mice. *Clin Cancer Res*. (2006) 12:5520–5. doi: 10.1158/1078-0432.CCR-06-0035
72. Hippen KL, Harker-Murray P, Porter SB, Merkel SC, Londer A, Taylor DK, et al. Umbilical cord blood regulatory T-cell expansion and functional effects of tumor necrosis factor receptor family members OX40 and 4-1BB expressed on artificial antigen-presenting cells. *Blood*. (2008) 112:2847–57. doi: 10.1182/blood-2008-01-132951
73. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T cell adoptive immunotherapy prevents acute leukemia relapse. *Blood*. (2014) 124:638–44. doi: 10.1182/blood-2014-03-564401
74. Parmar S, Liu X, Tung SS, Robinson SN, Rodriguez G, Cooper LJ, et al. Third-party umbilical cord blood-derived regulatory T cells prevent xenogenic graft-versus-host disease. *Cytotherapy*. (2014) 16:90–100. doi: 10.1016/j.jcyt.2013.07.009
75. Parmar S, Liu X, Najjar A, Shah N, Yang H, Yvon E, et al. *Ex vivo* fucosylation of third-party human regulatory T cells enhances anti-graft-versus-host disease potency *in vivo*. *Blood*. (2015) 125:1502–6. doi: 10.1182/blood-2014-10-603449
76. Joffre O, Gorsse N, Romagnoli P, Hudrisier D, van Meerwijk JP. Induction of antigen-specific tolerance to bone marrow allografts with $CD4^+CD25^+$ T lymphocytes. *Blood*. (2004) 103:4216–21. doi: 10.1182/blood-2004-01-0005
77. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, et al. Prevention of acute and chronic allograft rejection with $CD4^+CD25^+Foxp3^+$ regulatory T lymphocytes. *Nat Med*. (2008) 14:88–92. doi: 10.1038/nm1688
78. Hanash AM, Levy RB. Donor $CD4^+CD25^+$ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell transplantation. *Blood*. (2005) 105:1828–36. doi: 10.1182/blood-2004-08-3213
79. Steiner D, Brunicki N, Blazar BR, Bachar-Lustig E, Reisner Y. Tolerance induction by third-party "off-the-shelf" $CD4^+CD25^+$ Treg cells. *Exp Hematol*. (2006) 34:66–71. doi: 10.1016/j.exphem.2005.10.011
80. Pilat N, Baranyi U, Klaus C, Jaeckel E, Mpofu N, Wrba F, et al. Treg-therapy allows mixed chimerism and transplantation tolerance without cytoreductive conditioning. *Am J Transplant*. (2010) 10:751–62. doi: 10.1111/j.1600-6143.2010.03018.x

81. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. *Blood*. (2005) 105:4743–8. doi: 10.1182/blood-2004-10-3932
82. Hongo D, Tang X, Dutt S, Nador RG, Strober S. Interactions between NKT cells and Tregs are required for tolerance to combined bone marrow and organ transplants. *Blood*. (2012) 119:1581–9. doi: 10.1182/blood-2011-08-371948
83. Müller AM, Poyser J, Küpper NJ, Burnett C, Ko RM, Kohrt HE, et al. Donor hematopoiesis in mice following total lymphoid irradiation requires host T-regulatory cells for durable engraftment. *Blood*. (2014) 123:2882–92. doi: 10.1182/blood-2013-10-530212
84. Lowsky R, Takahashi T, Liu YP, Dejbakhsh-Jones S, Grumet FC, Shizuru JA, et al. Protective conditioning for acute graft-versus-host disease. *N Engl J Med*. (2005) 353:1321–31. doi: 10.1056/NEJMoa050642
85. Aversa F, Tabilio A, Velardi A, Cunningham I, Terenzi A, Falzetti F, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med*. (1998) 339:1186–93. doi: 10.1056/NEJM199810223391702
86. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol*. (2005) 23:3447–54. doi: 10.1200/JCO.2005.09.117
87. Mancusi A, Ruggeri L, Velardi A. Haploidentical hematopoietic transplantation for the cure of leukemia: from its biology to clinical translation. *Blood*. (2016) 128:2616–23. doi: 10.1182/blood-2016-07-730564
88. Kanakry CG, Fuchs EJ, Luznik L. Modern approaches to HLA-haploidentical blood or marrow transplantation. *Nat Rev Clin Oncol*. (2016) 13:132. doi: 10.1038/nrclinonc.2015.128
89. O'Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, Phelps M, et al. Nonmyeloablative bone marrow transplantation from partially HLA-mismatched related donors using posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. (2002) 8:377–86. doi: 10.1053/bbmt.2002.v8.pm12171484
90. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
91. Kanakry CG, Ganguly S, Zahurak M, Bolaños-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. *Sci Transl Med*. (2013) 5:211ra157. doi: 10.1126/scitranslmed.3006960
92. Ganguly S, Ross DB, Panoskaltsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, et al. Donor $CD4^+$ $Foxp3^+$ regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. *Blood*. (2014) 124:2131–41. doi: 10.1182/blood-2013-10-525873
93. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post- transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. *J Clin Invest*. (2019) 129:2357–73. doi: 10.1172/JCI124218
94. Russo A, Oliveira G, Berglund S, Greco R, Gambacorta V, Cieri N, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood*. (2018) 131:247–62. doi: 10.1182/blood-2017-05-780668
95. Roberto A, Di Vito C, Zaghi E, Mazza EMC, Capucetti A, Calvi M, et al. The early expansion of anergic NKG2Apos/CD56dim/CD16neg natural killer represents a therapeutic target in haploidentical hematopoietic stem cell transplantation. *Haematologica*. (2018) 103:1390–402. doi: 10.3324/haematol.2017.186619
96. Raiola AM, Dominietto A, di Grazia C, Lamparelli T, Gualandi F, Ibatici A, et al. Unmanipulated haploidentical transplants compared with other alternative donors and matched sibling grafts. *Biol Blood Marrow Transplant*. (2014) 20:1573–9. doi: 10.1016/j.bbmt.2014.05.029
97. Di Stasi A, Milton DR, Poon LM, Hamdi A, Rondon G, Chen J, et al. Similar transplantation outcomes for acute myeloid leukemia and myelodysplastic syndrome patients with haploidentical versus 10/10 human leukocyte antigen-matched unrelated and related donors. *Biol Blood Marrow Transplant*. (2014) 20:1975–81. doi: 10.1016/j.bbmt.2014.08.013
98. Ciurea SO, Zhang MJ, Bacigalupo AA, Bashey A, Appelbaum FR, Aljitawi OS, et al. Haploidentical transplant with posttransplant cyclophosphamide vs. matched unrelated donor transplant for acute myeloid leukemia. *Blood*. (2015) 126:1033–40. doi: 10.1182/blood-2015-04-639831
99. Kanate AS, Mussetti A, Kharfan-Dabaja MA, Ahn KW, DiGilio A, Beitinjaneh A, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors vs. HLA- matched unrelated donors. *Blood*. (2016) 127:938–47. doi: 10.1182/blood-2015-09-671834
100. Ghosh N, Karmali R, Rocha V, Ahn KW, DiGilio A, Hari PN, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors versus HLA-matched sibling donors: a center for international blood and marrow transplant research analysis. *J Clin Oncol*. (2016) 34:3141–9. doi: 10.1200/JCO.2015.66.3476
101. Bashey A, Zhang X, Jackson K, Brown S, Ridgeway M, Solh M, et al. Comparison of outcomes of hematopoietic cell transplants from T-replete haploidentical donors using post-transplantation cyclophosphamide with 10 of 10 HLA-A, -B, -C, -DRB1, and -DQB1 allele-matched unrelated donors and HLA-identical sibling donors: a multivariable analysis including disease risk index. *Biol Blood Marrow Transplant*. (2016) 22:125–33. doi: 10.1016/j.bbmt.2015.09.002
102. Dreger P, Sureda A, Ahn KW, Eapen M, Litovich C, Finel H, et al. PTCy-based haploidentical vs. matched related or unrelated donor reduced-intensity conditioning transplant for DLBCL. *Blood Adv*. (2019) 3:360–9. doi: 10.1182/bloodadvances.2018027748
103. Peccatori J, Forcina A, Clerici D, Crocchiolo R, Vago L, Stanghellini MT, et al. Sirolimus-based graft-versus-host disease prophylaxis promotes the *in vivo* expansion of regulatory T cells and permits peripheral blood stem cell transplantation from haploidentical donors. *Leukemia*. (2015) 29:396–405. doi: 10.1038/leu.2014.180
104. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*. (2011) 117:3921–8. doi: 10.1182/blood-2010-10-311894
105. Martelli MF, Di Ianni M, Ruggeri L, Pierini A, Falzetti F, Carotti A, et al. "Designed" grafts for HLA-haploidentical stem cell transplantation. *Blood*. (2014) 123:967–73. doi: 10.1182/blood-2013-10-531764
106. Rosenthal J, Wong J, Stein A, Qian D, Hitt D, Naeem H, et al. Phase 1/2 trial of total marrow and lymph node irradiation to augment reduced-intensity transplantation for advanced hematologic malignancies. *Blood*. (2011) 117:309–15. doi: 10.1182/blood-2010-06-288357
107. Pierini A, Ruggeri L, Carotti A, Falzetti F, Piccinelli S, Saldi S, et al. The "ultimate" haploidentical transplantation for the elderly with high-risk acute myeloid leukemia. *Bone Marrow Transplant*. (2019) 54(Suppl. 2):803–5. doi: 10.1038/s41409-019-0618-x
108. MacDonald KN, Piret JM, Levings MK. Methods to manufacture regulatory T cells for cell therapy. *Clin Exp Immunol*. (2019) 197:52–63. doi: 10.1111/cei.13297
109. Trzonkowski P, Bieniaszewska M, Juścinska J, Dobyszuk A, Krzystyniak A, Marek N, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human *ex vivo* expanded $CD4^+CD25^+CD127-$ T regulatory cells. *Clin Immunol*. (2009) 133:22–6. doi: 10.1016/j.clim.2009.06.001
110. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of *ex vivo* expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. (2011) 117:1061–70. doi: 10.1182/blood-2010-07-293795
111. Theil A, Tuve S, Oelschlägel U, Maiwald A, Döhler D, Oßmann D, et al. Adoptive transfer of allogeneic regulatory T cells into patients with chronic graft-versus-host disease. *Cytotherapy*. (2015) 17:473–86. doi: 10.1016/j.jcyt.2014.11.005
112. Del Papa B, Ruggeri L, Urbani E, Baldoni S, Cecchini D, Zei T, et al. Clinical-grade-expanded regulatory T cells prevent graft-versus-host disease while allowing a powerful T cell-dependent graft-versus-leukemia effect in murine models. *Biol Blood Marrow Transplant*. (2017) 23:1847–51. doi: 10.1016/j.bbmt.2017.07.009
113. Hippen KL, Merkel SC, Schirm DK, Sieben CM, Sumstad D, Kadidlo DM, et al. Massive *ex vivo* expansion of human natural regulatory T cells (T(regs))

with minimal loss of *in vivo* functional activity. *Sci Transl Med*. (2011) 3:83ra41. doi: 10.1126/scitranslmed.3001809
114. Brunstein CG, Miller JS, McKenna DH, Hippen KL, DeFor TE, Sumstad D, et al. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood*. (2016) 127:1044–51. doi: 10.1182/blood-2015-06-653667
115. McKenna DH Jr, Sumstad D, Kadidlo DM, Batdorf B, Lord CJ, Merkel SC, et al. Optimization of cGMP purification and expansion of umbilical cord blood-derived T-regulatory cells in support of first-in-human clinical trials. *Cytotherapy*. (2017) 19:250–62. doi: 10.1016/j.jcyt.2016.10.011
116. Meyer EH, Laport G, Xie BJ, MacDonald K, Heydari K, Sahaf B, et al. Transplantation of donor grafts with defined ratio of conventional and regulatory T cells in HLA-matched recipients. *JCI Insight*. (2019) 4:127244. doi: 10.1172/jci.insight.127244
117. Florek M, Schneidawind D, Pierini A, Baker J, Armstrong R, Pan Y, et al. Freeze and thaw of $CD4^+CD25^+Foxp3^+$ regulatory T cells results in loss of CD62L expression and a reduced capacity to protect against graft-versus-host disease. *PLoS ONE*. (2015) 10:e0145763. doi: 10.1371/journal.pone.0145763
118. Saadoun D, Rosenzwajg M, Joly F, Six A, Carrat F, Thibault V, et al. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med*. (2011) 365:2067–77. doi: 10.1056/NEJMoa1105143
119. Klatzmann D, Abbas AK. The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat Rev Immunol*. (2015) 15:283–94. doi: 10.1038/nri3823
120. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP III, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med*. (2011) 365:2055–66. doi: 10.1056/NEJMoa1108188
121. Koreth J, Kim HT, Jones KT, Lange PB, Reynolds CG, Chammas MJ, et al. Efficacy, durability, and response predictors of low-dose interleukin-2 therapy for chronic graft-versus-host disease. *Blood*. (2016) 128:130–7. doi: 10.1182/blood-2016-02-702852
122. Matsuoka K, Koreth J, Kim HT, Bascug G, McDonough S, Kawano Y, et al. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med*. (2013) 5:179ra43. doi: 10.1126/scitranslmed.3005265
123. Cutler CS, Koreth J, Ritz J. Mechanistic approaches for the prevention and treatment of chronic GVHD. *Blood*. (2017) 129:22–9. doi: 10.1182/blood-2016-08-686659
124. Belizaire R, Kim HT, Poryanda SJ, Mirkovic NV, Hipolito E, Savage WJ, et al. Efficacy and immunologic effects of extracorporeal photopheresis plus interleukin-2 in chronic graft-versus-host disease. *Blood Adv*. (2019) 3:969–79. doi: 10.1182/bloodadvances.2018029124
125. Kennedy-Nasser AA, Ku S, Castillo-Caro P, Hazrat Y, Wu MF, Liu H, et al. Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity. *Clin Cancer Res*. (2014) 20:2215–25. doi: 10.1158/1078-0432.CCR-13-3205
126. Abbas AK, Trotta E, Simeonov DR, Marson A, Bluestone JA. Revisiting IL-2: biology and therapeutic prospects. *Sci Immunol*. (2018) 3:eaat1482. doi: 10.1126/sciimmunol.aat1482
127. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science*. (2006) 311:1924–7. doi: 10.1126/science.1122927
128. Trotta E, Bessette PH, Silveria SL, Ely LK, Jude KM, Le DT, et al. A human anti-IL-2 antibody that potentiates regulatory T cells by a structure-based mechanism. *Nat Med*. (2018) 24:1005–14. doi: 10.1038/s41591-018-0070-2
129. Spangler JB, Trotta E, Tomala J, Peck A, Young TA, Savvides CS, et al. Engineering a single-agent cytokine/antibody fusion that selectively expands regulatory T cells for autoimmune disease therapy. *J Immunol*. (2018) 201:2094–106. doi: 10.4049/jimmunol.1800578
130. Ward NC, Yu A, Moro A, Ban Y, Chen X, Hsiung S, et al. Review IL-2/CD25: a long-acting fusion protein that promotes immune tolerance by selectively targeting the IL-2 receptor on regulatory T cells. *J Immunol*. (2018) 201:2579–92. doi: 10.4049/jimmunol.1800907
131. Tchao N, Gorski KS, Yuraszeck T, Sohn SJ, Ishida K, Wong H, et al. Amg 592 is an investigational IL-2 mutein that induces highly selective expansion of regulatory T cells. *Blood*. (2017) 130 (Suppl. 1): 696. doi: 10.1182/blood.V126.23.919.919
132. Peterson LB, Bell CJM, Howlett SK, Pekalski ML, Brady K, Hinton H, et al. A long-lived IL-2 mutein that selectively activates and expands regulatory T cells as a therapy for autoimmune disease. *J Autoimmun*. (2018) 95:1–14. doi: 10.1016/j.jaut.2018.10.017
133. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
134. Levine JE. Implications of TNF-α in the pathogenesis and management of GVHD. *Int J Hematol*. (2011) 93:571–7. doi: 10.1007/s12185-011-0803-1
135. Yalniz FF, Hefazi M, McCullough K, Litzow MR, Hogan WJ, Wolf R, et al. Safety and efficacy of infliximab therapy in the setting of steroid-refractory acute graft-versus-host disease. *Biol Blood Marrow Transplant*. (2017) 23:1478–84. doi: 10.1016/j.bbmt.2017.05.001
136. Patriarca F, Sperotto A, Damiani D, Morreale G, Bonifazi F, Olivieri A, et al. Infliximab treatment for steroid-refractory acute graft-versus-host disease. *Haematologica*. (2004) 11:1352–9.
137. Chen X, Bäumel M, Männel DN, Howard OM, Oppenheim JJ. Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse $CD4^+CD25^+$ T regulatory cells. *J Immunol*. (2007) 179:154–61. doi: 10.4049/jimmunol.179.1.154
138. Chen X, Subleski JJ, Kopf H, Howard OM, Männel DN, Oppenheim JJ. Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse $CD4^+CD25^+FoxP3^+$ T regulatory cells: applicability to tumor-infiltrating T regulatory cells. *J Immunol*. (2008) 180:6467–71. doi: 10.4049/jimmunol.180.10.6467
139. Chen X, Subleski JJ, Hamano R, Howard OM, Wiltrout RH, Oppenheim JJ. Co-expression of TNFR2 and CD25 identifies more of the functional $CD4^+FOXP3^+$ regulatory T cells in human peripheral blood. *Eur J Immunol*. (2010) 40:1099–106. doi: 10.1002/eji.200940022
140. Okubo Y, Mera T, Wang L, Faustman DL. Homogeneous expansion of human T-regulatory cells via tumor necrosis factor receptor 2. *Sci Rep*. (2013) 3:3153. doi: 10.1038/srep03153
141. Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, et al. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol*. (1998) 160:943–52.
142. Mahmud SA, Manlove LS, Schmitz HM, Xing Y, Wang Y, Owen DL, et al. Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat Immunol*. (2014) 15:473–81. doi: 10.1038/ni.2849
143. Leclerc M, Naserian S, Pilon C, Thiolat A, Martin GH, Pouchy C, et al. Control of GVHD by regulatory T cells depends on TNF produced by T cells and TNFR2 expressed by regulatory T cells. *Blood*. (2016) 128:1651–9. doi: 10.1182/blood-2016-02-700849
144. Pierini A, Strober W, Moffett C, Baker J, Nishikii H, Alvarez M, et al. TNF-α priming enhances $CD4^+FoxP3^+$ regulatory T-cell suppressive function in murine GVHD prevention and treatment. *Blood*. (2016) 128:866–71. doi: 10.1182/blood-2016-04-711275
145. Chopra M, Biehl M, Steinfatt T, Brandl A, Kums J, Amich J, et al. Exogenous TNFR2 activation protects from acute GvHD via host T reg cell expansion. *J Exp Med*. (2016) 213:1881–900. doi: 10.1084/jem.20151563
146. Mancusi A, Piccinelli S, Velardi A, Pierini A. The Effect of TNF-α on regulatory T cell function in graft-versus-host disease. *Front Immunol*. (2018) 9:356. doi: 10.3389/fimmu.2018.00356
147. Mancusi A, Alvarez M, Piccinelli S, Velardi A, Pierini A. TNFR2 signaling modulates immunity after allogeneic hematopoietic cell transplantation. *Cytokine Growth Factor Rev*. (2019) 47:54–61. doi: 10.1016/j.cytogfr.2019.05.001
148. Chen X, Hamano R, Jubleski JJ, Hurwitz AA, Howard OM, Oppenheim JJ. Expression of costimulatory TNFR2 induces resistance of $CD4^+FoxP3^-$ conventional T cells to suppression by $CD4^+FoxP3^+$ regulatory T cells. *J Immunol*. (2010) 185:174–82. doi: 10.4049/jimmunol.0903548
149. Schreiber TH, Wolf D, Bodero M, Gonzalez L, Podack ER. T cell costimulation by TNFR superfamily (TNFRSF)4 and TNFRSF25 in the context of vaccination. *J Immunol*. (2012) 189:3311–8. doi: 10.4049/jimmunol.1200597

150. Khan SQ, Tsai MS, Schreiber TH, Wolf D, Deyev VV, Podack ER. Cloning, expression, and functional characterization of TL1A-Ig. *J Immunol*. (2013) 190:1540–50. doi: 10.4049/jimmunol.1201908
151. Kim BS, Nishikii H, Baker J, Pierini A, Schneidawind D, Pan Y, et al. Treatment with agonistic DR3 antibody results in expansion of donor Tregs and reduced graft-versus-host disease. *Blood*. (2015) 126:546–57. doi: 10.1182/blood-2015-04-637587
152. Nishikii H, Kim BS, Yokoyama Y, Chen Y, Baker J, Pierini A, et al. DR3 signaling modulates the function of Foxp3$^+$ regulatory T cells and the severity of acute graft-versus-host disease. *Blood*. (2016) 128:2846–58. doi: 10.1182/blood-2016-06-723783
153. Wolf D, Barreras H, Bader CS, Copsel S, Lightbourn CO, Pfeiffer BJ, et al. Marked *in vivo* donor regulatory T cell expansion via interleukin-2 and TL1A-Ig stimulation ameliorates graft-versus-host disease but preserves graft-versus-leukemia in recipients after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2017) 23:757–66. doi: 10.1016/j.bbmt.2017.02.013
154. Copsel S, Wolf D, Kale B, Barreras H, Lightbourn CO, Bader CS, et al. Very low numbers of CD4$^+$ FoxP3$^+$ tregs expanded in donors via TL1A-Ig and low-dose IL-2 exhibit a distinct activation/functional profile and suppress GVHD in a preclinical model. *Biol Blood Marrow Transplant*. (2018) 24:1788–94. doi: 10.1016/j.bbmt.2018.04.026
155. Mavers M, Simonetta F, Nishikii H, Ribado JV, Maas-Bauer K, Alvarez M, et al. Activation of the DR3-TL1A axis in donor mice leads to regulatory T cell expansion and activation with reduction in graft-versus-host disease. *Front Immunol*. (2019) 10:1624. doi: 10.3389/fimmu.2019.01624
156. Copsel SN, Lightbourn CO, Barreras H, Lohse I, Wolf D, Bader CS, et al. BET Bromodomain inhibitors which permit Treg function enable a combinatorial strategy to suppress GVHD in pre-clinical allogeneic HSCT. *Front Immunol*. (2019) 9:3104. doi: 10.3389/fimmu.2018.03104
157. Dawson NAJ, Vent-Schmidt J, Levings MK. Engineered tolerance: tailoring development, function, and antigen-specificity of regulatory T cells. *Front Immunol*. (2017) 8:1460. doi: 10.3389/fimmu.2017.01460
158. MacDonald KG, Hoeppli RE, Huang Q, Gillies J, Luciani DS, Orban PC, et al. Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. *J Clin Invest*. (2016) 126:1413–24. doi: 10.1172/JCI82771
159. Noyan F, Zimmermann K, Hardtke-Wolenski M, Knoefel A, Schulde E, Geffers R, et al. Prevention of allograft rejection by use of regulatory T cells with an MHC-specific chimeric antigen receptor. *Am J Transplant*. (2017) 17:917–30. doi: 10.1111/ajt.14175
160. Pierini A, Iliopoulou BP, Peiris H, Pérez-Cruz M, Baker J, Hsu K, et al. T cells expressing chimeric antigen receptor promote immune tolerance. *JCI Insight*. (2017) 2:92865. doi: 10.1172/jci.insight.92865
161. Zeiser R, Blazar BR. Acute graft-versus-host disease biology, prevention and therapy. *N Engl J Med*. (2017) 377: 2167–79. doi: 10.1056/NEJMra1609337

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REVIEW
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Cyclophosphamide-Induced Tolerance in Allogeneic Transplantation: From Basic Studies to Clinical Application

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Immune tolerance against alloantigens plays an important role in the success of clinical organ and allogeneic hematopoietic stem cell transplantation. The mechanisms of immune tolerance to alloantigens have gradually been elucidated over time. Although there have been numerous reports to date on the induction of tolerance to alloantigens, the establishment of mixed chimerism is well-known to be crucial in the induction and maintenance of immune tolerance for either of the methods. Since the early 1980s, the murine system of cyclophosphamide (Cy)-induced tolerance has also been examined extensively. The present review focuses on studies conducted on Cy-induced immune tolerance. Clinical data of patients with allogeneic transplantation suggest that the posttransplant Cy method to induce immune tolerance has been successfully translated from basic studies into clinical practice.

Keywords: tolerance, cyclophosphamide, graft-vs.-host disease, allogeneic hematopoietic stem cell transplantation, kidney transplantation

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INTRODUCTION

Donor availability remains a limiting factor for success of allogeneic hematopoietic stem cell transplantation (allo-HSCT). A suitable human leukocyte antigen (HLA)-matched sibling or unrelated donor cannot be identified in time for about half of transplant recipients. On the other hand, an HLA-haploidentical donor can be identified rapidly in most cases. However, although rapid donor availability is the major advantage of HLA-haploidentical allo-HSCT, the obstacles of HLA-haploidentical allo-HSCT with a T-cell-replete graft include a high incidence of severe graft-vs.-host disease (GVHD), resulting in an increased incidence of non-relapse mortality at ∼50% in early trials (1, 2). Certainly, *ex vivo* depletion of graft T cells reduces the risk of severe GVHD after HLA-haploidentical allo-HSCT; however, it is associated with an increased risk of engraftment failure and severe infections. Similarly, opportunistic infections which are associated with the suppression of cell-mediated immunity have now become pressing issues related to kidney transplantation, although the advances in immunosuppressants such as cyclosporine and tacrolimus have resulted in decreasing incidence of graft rejection in organ transplantation (3). These are as a result of non-specific immunosuppression, which suppresses the function of T cells in general.

To reduce the incidence of GVHD in HLA-haploidentical allo-HSCT through the donor-specific induction of immune tolerance in host or to avoid graft rejection in organ transplantation

through the recipient-specific induction of immune tolerance against donor graft is the eventual goal for success of these allogeneic transplantation. This can be approached by selectively depleting alloreactive T cells; however, there is still no established method to achieve this goal. Recently, several research groups developed a method with high doses of cyclophosphamide (Cy) administered just after allogeneic transplantation (4, 5). In this article, we will provide a general outline of this topic, including a history of the basic research conducted to date.

WHAT IS IMMUNE TOLERANCE?

Herein, we mainly describe immune tolerance to alloantigens (donor antigens) within the overall context of immune tolerance. First, however, we must discuss the induction and maintenance of tolerance to self-antigens. Tolerance against self-antigens is crucial in preventing the development of autoimmune diseases. Clonal deletion by eliminating autoreactive T cells has been proposed as the mechanism for the induction of tolerance. Tolerance has been clarified through the specific relationship between superantigens and certain Vβ segments of the T-cell receptor. In the late 1980s, clonal deletion in the thymus was shown in a mouse model with superantigens (e.g., Mls^a antigens), which can combine with major histocompatibility complex (MHC) antigen class II molecules and can respond strongly to T cells via the certain Vβ segments (e.g., Vβ6). In this mouse model with self-Mls^a antigens, specific Vβ6-positive T cells are eliminated in the periphery (6, 7). Indeed, these Vβ6-positive T cells were shown to be depleted during their differentiation in the thymus (central tolerance) (8). This was the first report of a method to explain the induction of self-tolerance through clonal deletion. Although central tolerance via clonal deletion is considered to be sufficient, they cannot control self-reactivity completely. Peripheral deletion mediated predominately via a Fas/FasL mechanism is one mechanism by which the immune system eliminates self-reactive T cells that escaped from central tolerance. Other mechanisms have been proposed for the induction and maintenance of self-tolerance. These include paralyzing autoreactive T cells (clonal anergy) and continuously suppressing autoreactive T cells by way of suppressor T cells. By these peripheral tolerances via regulatory T cells (Tregs) and cytokines, self-reactive T cells are rendered anergic even after encountering self-antigens outside of the thymus.

Immune tolerance against alloantigens plays an important role in the success of clinical organ and hematopoietic stem cell transplantation. There have been many reports of methods to date for the induction of tolerance to alloantigens (e.g., induction of immune tolerance in neonates, induction of tolerance using irradiation, induction of tolerance using monoclonal antibodies, and drug-induced immune tolerance). Although the establishment of mixed chimerism, in which donor cells are found at a certain rate in the recipient's body, is widely known to be crucial in the induction and maintenance of immune tolerance for either of the methods (9), MacDonald et al. demonstrated that the induction of immune tolerance in neonates was due to the intrathymic clonal deletion of alloantigen-reactive T cells (10). Especially after Starzl et al. reported that a microchimerism was established in some patients after liver transplantation in whom immunosuppressive treatment could be discontinued without the occurrence of graft rejection (11, 12), much work has been focused on how to induce immune tolerance by establishing chimerism in the field of clinical organ transplantation (13, 14). In addition, drug-induced immune tolerance with Cy was effective in xenotransplantation against B cells that produce xenoreactive antibodies (15). Thus, the mechanisms of immune tolerance to alloantigens have gradually been elucidated over time. Since the early 1980s, Professor Kikuo Nomoto's laboratory in the Department of Immunology, Medical Institute of Bioregulation, Kyushu University has extensively reexamined and developed a murine system of Cy-induced tolerance to show central and peripheral clonal deletion (4).

CYCLOPHOSPHAMIDE-INDUCED IMMUNE TOLERANCE

Cy is a chemotherapeutic agent. Since Cy has been in clinical rotation for about 50 years, there is much experience to draw on for using this agent in the treatment of cancer and autoimmune diseases. Besides chemotherapeutic effects, Cy has immunosuppressive as well as immunomodulatory abilities. In 1963, Berenbaum and Brown first demonstrated the effects of Cy on the allogeneic response (16). Cy (200 mg/kg) was intraperitoneally administered to mice before or after an MHC-mismatched allogeneic skin graft (17). While control mice lost the allogeneic skin graft after ∼14 days, mice treated with Cy revealed delayed skin graft rejection. When a single dose of Cy just after allogeneic skin grafting (day 0) was administered between days 0 and 4, it was shown to be more effective in prolonging graft survival compared to Cy use on day 6 or between days −4 and 0. Santos and Owens reported that Cy reduced the incidence and severity of GVHD when Cy was given on days 2, 3, and 5 after the infusion of allogeneic spleen cells in rats (18). From the extensive studies on the cells-followed-by-chemotherapeutic drugs system by many investigators, the optimal timing of chemotherapeutic drug use for the induction of tolerance is 1–4 days after antigen exposure; however, the different dose, timing, and cumulative exposure of Cy are critical for its efficacy in preventing GVHD (19). In addition, chemotherapeutic drugs such as 6-mercaptopurine, methotrexate, and 5-fluorouracil may be useful for promoting the success of the cells-followed-by-chemotherapeutic drug system, Cy is known to have the greatest tolerance induction potential among these chemotherapeutic drugs (20, 21). Treatment of Cy with other immunosuppressive drugs such as steroids, cyclosporine, and tacrolimus before or together with the allogeneic cell infusion impaired the development of the tolerance induction because the cell proliferation was inhibited as a result of these drugs being used for pretreatment (22).

Based on these results, the Nomoto's laboratory developed a method, the so-called "cells-followed-by-Cy system" for inducing tolerance to allogeneic grafts (4, 23). Here, the method of the

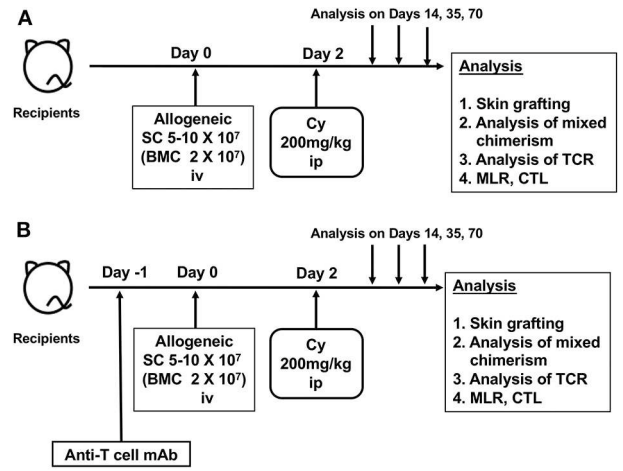

FIGURE 1 | The schema of cyclophosphamide-induced tolerance model. **(A)** Original protocol of cyclophosphamide-induced tolerance model. **(B)** Modified protocol using anti-T-cell monoclonal antibody. SC, spleen cell; BMC, bone marrow cell; Cy, cyclophosphamide; mAb, monoclonal antibody; ip, intraperitoneal injection; iv, intravenous injection; MLR, mixed lymphocyte reaction analysis; CTL, cytotoxic T-lymphocyte analysis; TCR, T-cell-receptor analysis.

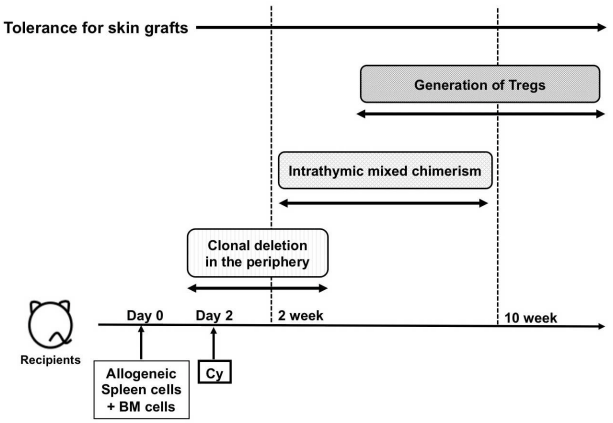

FIGURE 2 | The flow of the three major mechanisms of cyclophosphamide-induced tolerance. Cy, cyclophosphamide; BM, bone marrow; PBSCT; Treg, regulatory T cells.

Cy-induced immune tolerance that we have reported on to date consists of a very simple process of administering 5–10 $\times$ 10^7 allogeneic spleen cells (bone marrow cells were later added) intravenously 2 days before intraperitoneally administering Cy at a dose of 150–200 mg/kg (**Figure 1A**). The most distinctive feature is the administration of Cy after rather than before the administration of alloantigens (24–26). Furthermore, the "cells-followed-by-Cy system" in combination with using anti-T-cell monoclonal antibody and low-dose total body irradiation (TBI) on day −1 could induce a profound tolerance with sustained mixed chimerism to skin or other solid organs in various mouse combinations with differing MHCs and minor antigens (5, 27–29) (**Figure 1B**). Thus, through the extensive efforts of basic researches, this "cells-followed-by-Cy system" has been also referred to as "posttransplant Cy (PTCy)" in the clinical field of allo-HSCT and solid organ transplantation.

MECHANISMS OF CYCLOPHOSPHAMIDE-INDUCED IMMUNE TOLERANCE

Using the aforementioned correlations between Mls^a antigens and Vβ6-positive T cells, the Nomoto's laboratory investigated the mechanism of immune induction and maintenance of tolerance to alloantigens through Cy-induced immune tolerance and identified the following three distinct and sequential mechanisms in MHC-matched setting. The first mechanism was the deletion of alloantigen-stimulated T cells after Cy treatment in the periphery (30, 31). The second mechanism was intrathymic clonal deletion of donor-reactive T cells, which was strongly associated with mixed chimerism in the thymus during the maintenance phase. The third mechanism was the generation of suppressor T cells in the late stage of the tolerance. The flows of the three mechanisms over time are summarized in **Figure 2**.

Clonal Deletion of Donor-Reactive T Cells During the Induction of Tolerance

When AKR/J (H-2k, Mls^a, Thy-1.1) is used as the donor and C3H/He (H-2k, Mls^b, Thy-1.2) is used as the recipient, long-term engraftment of AKR/J skin allografts can be induced through Cy-induced immune tolerance (32). When AKR/J splenocytes were administered to recipient C3H/He and Cy was administered 2 days later, the number of CD4-positive Vβ6-positive T cells with strong reactivity to donor Mls^a antigens selectively decreased from an early stage and were remarkably decreased at 5 weeks after Cy administration. No such changes were observed in control Vβ8-positive T cells, indicating that the findings were specific to Vβ6-positive T cells. In addition, since CD8-positive T cells were less reactive to Mls^a antigens in Vβ6-positive T cells, a response similar to the one observed in CD4-positive T cells was not seen. These results indicate that alloreactive T cells are selectively eliminated after Cy in the periphery. The clonal deletion mechanism acts on mature T cells, which are outside of the bone marrow and thymus. In addition, clonal deletion at the time of induction of tolerance was also observed in recipient-reactive T cells from treated donors, and an involvement in the prevention of GVHD was also revealed through an evaluation of the transition of Vβ3-positive T cells from donor AKR/J reactive to Mls-2^a antigens of recipient C3H/He.

Intrathymic Mixed Chimerism in the Maintenance Phase

In AKR/J→C3H/He combinations, while Thy-1.1-positive T cells from donor AKR/J were observed in peripheral lymph nodes beginning immediately after the induction of tolerance, Thy-1.1-positive T cells from the donor AKR/J were not observed in the thymus of recipient C3H/He on day 14 after Cy administration, and Vβ6-positive T cells reactive to donor Mls^a antigens were found at normal levels (32). However, by day 35 after the

administration of Cy, Thy-1.1-positive T cells from donor AKR/J were found in the thymus of recipient C3H/He, suggesting that the hematopoietic cells in donor AKR/J splenocytes had differentiated and matured in the thymus of recipient C3H/He. In the thymus that was in such a chimeric state, there was clonal deletion of Vβ6-positive T cells reactive to donor Mls^a antigens. This indicates that chimerism is also established at the antigen-presenting cell level in recipient thymus. Clonal deletion of Vβ6-positive T cells was observed in either CD4- or CD8-positive mature thymic T cells, unlike the aforementioned peripheral clonal deletion, to allow for the negative selection of CD4- and CD8-positive immature thymocytes in the thymus (10, 33).

Generation of Tolerogen-Specific Regulatory T Cells in the Late Maintenance Phase

Although the clonal destruction of alloreactive T cells is thought to be the dominant mechanism of Cy-induced tolerance, it is insufficient to explain peripheral tolerance in the Cy-induced tolerance system. Tregs also have an important role in Cy-induced tolerance system (34–37). In DBA/2 ($H\text{-}2^d$, Mls^a)→BALB/c ($H\text{-}2^d$, Mls^b) combinations, intrathymic T-cell chimerism disappeared by day 100 following the induction of tolerance in some recipient BALB/c mice and, as a consequence, the intrathymic clonal deletion of Vβ6-positive T cells reactive to donor Mls^a antigens was also disrupted, with the regeneration of Vβ6-positive T cells observed in peripheral lymph nodes (38). This suggests that chimerism was also lost at the level of antigen-presenting cells in the thymus. Nevertheless, the skin allografts remained engrafted. Therefore, the presence or absence of the involvement of Tregs was evaluated as a mechanism of maintaining tolerance at this stage. The adoptive transfer of splenocytes from mice engrafted with donor skin allografts into syngeneic mice irradiated with low doses of radiation followed by the grafting of donor skin allografts on the following day revealed the presence of donor antigen-specific Tregs (39), with CD8-positive T cells being predominantly found in this combination. Tregs were insufficient at day 14 to prevent skin allograft rejection upon transfer. In addition, the presence of CD4-positive Tregs has also been observed among mice differing only in terms of class II antigens (40).

Because MHC-matched murine skin-allografting models with Mls antigens were highly contextual, the exact association of these three mechanisms to how PTCy prevents GVHD in allo-HSCT still remains unclear. Donor Tregs, which are resistant to PTCy via aldehyde dehydrogenase expression, are necessary for protection against GVHD (35, 37). A recent report showed that PTCy did not eliminate alloreactive T cells and the thymus was not necessary for efficacy of PTCy in T-cell replete, MHC-haploidentical, murine allo-HSCT model (B6C3F1→B6D2F1), whereas PTCy impaired the function of alloreactive T cells and the rapid recovery of Tregs played an important role in suppressive mechanisms of GVHD (36). In four other models including one of the same MHC-matched strain combinations as used in the skin allografting models, PTCy also did not eliminate alloreactive T cells. Rather, PTCy induced alloreactive T-cell functional impairment over time through the suppressive mechanism with rapid recovery of alloantigen-specific Tregs (36). In addition to the role of Tregs, clonal anergy was suggested to be involved in the maintenance of tolerance in the late phase of maintenance (41, 42), although this did not appear in allo-HSCT (36). In the early stage of induction of Cy-induced tolerance, observing higher chimerism of donor-derived cells in the recipient periphery was also found to be important in inducing a higher level of tolerance (43). The differential influence of Cy on each subset of T cells has been also reported. Among T cells spared by Cy treatment, naive-derived memory stem T cells (44–47) that can differentiate into various memory T cells are the most abundant T-cell population in the early period following PTCy haploidentical allo-HSCT and play an important role in immune reconstitution in the long term after transplantation (48, 49). Therefore, further understanding of tolerance induction by PTCy can be put in perspective in future studies.

CLINICAL APPLICATION OF CYCLOPHOSPHAMIDE-INDUCED IMMUNE TOLERANCE

The outline of the historical background on underlying mechanisms in Cy-induced immune tolerance has hereby been provided. Of note, however, Cy-induced tolerance is now also receiving great attention in two clinical fields: allo-HSCT and kidney transplantation.

Clinical Application in Allo-HSCT

As previously described, a characteristic feature of the PTCy method is the fact that Cy is given after rather than before the administration of alloantigens. Especially in the field of HLA-haploidentical allo-HSCT, this method has garnered significant attention with respect to good engraftment and low incidence of GVHD. The Johns Hopkins University group has previously successfully achieved tolerance and mixed hematopoietic chimerism in mice treated with fludarabine (Flu) and 200 cGy TBI, transplanted with 10 million marrow cells, and given Cy 200 mg/kg intraperitoneally on day 3 (5). This method reduced the incidence and severity of GVHD in an MHC-mismatched combination. These promising results provided the rationale to conduct a clinical trial of PTCy HLA-haploidentical allo-HSCT for patients with poor prognoses for hematological malignancies (50–52) and non-neoplastic hematological diseases (53).

Based on these results, a phase I/II clinical trial of haploidentical bone marrow transplantation for hematological malignancies was initiated in 1999. Thirteen patients with hematological malignancies received conditioning with Flu (30 mg/sqm/day from days −6 to −2) and TBI (200 cGy on day −1). All patients received Cy at a single dose of 50 mg/kg on day 3 with tacrolimus and mycophenolate mofetil from day 4 as a GVHD prophylaxis (51). Of the first three patients, two conditioned without Cy developed engraftment failure in the phase I portion. Therefore, Cy was added to the conditioning

regimen at a total dose of 29 mg/kg given on days −5 and −6 in the next 10 patients, and 8 could achieve engraftment. Acute GVHD developed in six of these eight engrafted patients during the phase II portion and responded well to treatment. Results of the first 13 patients were reported as a proof-of-principle report in 2002 (51). Clinical outcomes of 68 patients in phase II trials were also reported in 2008 (52). In this trial, the dose of administered Cy was modified by increasing the total dose of PTCy to 100 mg/kg given over days 3 and 4 to decrease in the incidence of GVHD. Among 68 patients, 40 patients received the total dose of PTCy with 50 mg/kg on days +3 and +4, and 28 patients received 50 mg/kg on day +3. Primary engraft failure occurred in 13% of patients. The median times for neutrophil and platelet recovery were 15 and 24 days, respectively. There was no difference in the incidence of severe acute GVHD between one or two doses of PTCy with 34% of cumulative incidences being of grades II–IV and 6% being of grades III–IV acute GVHD. The cumulative incidences of non-relapse mortality and relapse at 1 year were 15 and 51%, respectively.

Thus, PTCy as GVHD prophylaxis has been developed initially for haploidentical bone marrow transplantation after non-myeloablative conditioning (**Figure 3A**); however, myeloablative conditioning or peripheral blood stem cells as the graft source have been successfully used in several studies (54–56). Among the many platforms used with PTCy, an example of a myeloablative approach with peripheral blood stem cells is shown in **Figure 3B**. In addition, recent studies demonstrated that PTCy could be applied even in HLA-matched allo-HSCT, and donor type might no longer be a significant predictor in the era of PTCy (57–59). Consequentially, allo-HSCT with PTCy has spread rapidly worldwide.

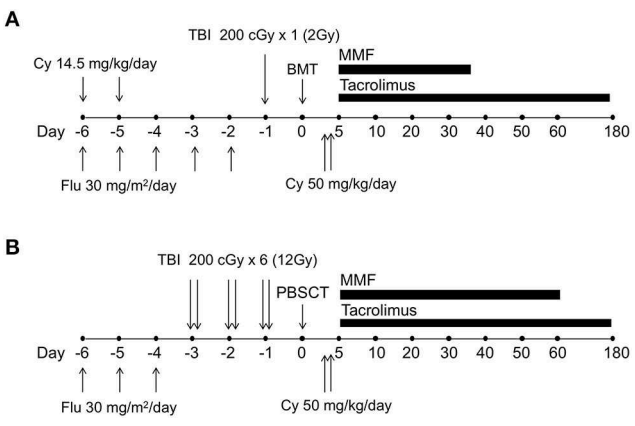

FIGURE 3 | The schema of human leukocyte antigen (HLA)-haploidentical transplantation with posttransplantation cyclophosphamide. **(A)** The schema of non-myeloablative, HLA-haploidentical bone marrow transplantation with posttransplantation cyclophosphamide, which was originally developed by the Johns Hopkins University group. **(B)** One example schema for myeloablative, HLA-haploidentical peripheral blood stem cell transplantation with posttransplantation cyclophosphamide. Cy, cyclophosphamide; Flu, fludarabine; TBI, total body irradiation; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; MMF, mycophenolate mofetil.

Clinical Application to Kidney Transplantation

Clinical trials to induce renal allograft tolerance with allogeneic stem cell have been reported from three centers: Northwestern University, Stanford University, and Massachusetts General Hospital in the United States (60). Aside from the methods inducing tolerance by PTCy, Stanford University and Massachusetts General Hospital groups have been using their specific approaches to induce renal allograft tolerance (61). Researchers at Stanford University group used allo-HSCT with total lymphoid irradiation and rabbit antithymocyte globulin to induce mixed chimerism. In their experience, durable or transient chimerism was induced in HLA-matched transplant recipients, and immunosuppressive agents were withdrawn in ∼70% of the patients; however, induction of chimerism has been difficult in HLA-mismatched transplant recipients, and no recipients has achieved complete discontinuation of immunosuppression. The Massachusetts General Hospital group developed the conditioning regimen for HLA-mismatched kidney transplantation, which included Cy (60 mg/kg on days −5 and −4), thymic irradiation, anti-CD2 monoclonal antibody, and posttransplant calcineurin inhibitors administration (13, 14). In a revised regimen, low-dose TBI replaced Cy, and rituximab was added. Of the 10 recipients enrolled in the studies, all developed transient mixed chimerism and immunosuppression was discontinued in eight patients. After a follow-up period of 7–14 years, four patients still could discontinue immunosuppression completely (62).

The Northwestern University group reported results from a recent clinical trial involving patients undergoing living-donor kidney transplantation using the regimen shown in **Figure 4**, where stable donor-derived chimerism was able to be induced in 19 out of 31 recipients and immunosuppressive agents could also be discontinued (60, 63). In this report, patients were transplanted from six of six HLA-matched related to zero of six HLA-matched unrelated donor. Twelve subjects had unrelated

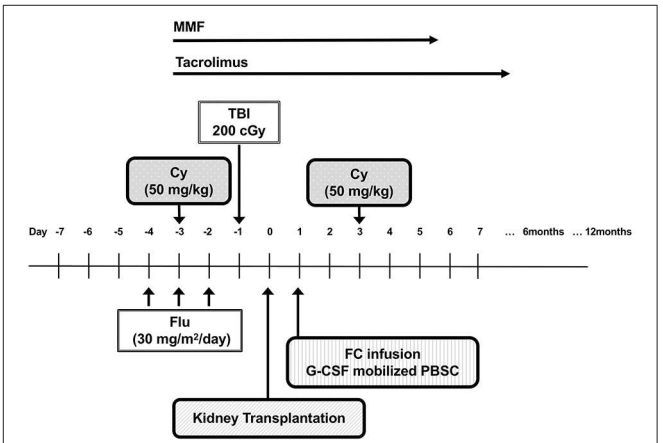

FIGURE 4 | The schema of living-donor kidney transplantation with posttransplantation cyclophosphamide. Cy, cyclophosphamide; Flu, fludarabine; TBI, total body irradiation; FCs, facilitating cells; MMF, mycophenolate mofetil002E.

and 19 had related donors. The patients are conditioned with Flu (30 mg/sqm/day on days −5, −4, −3), Cy (50 mg/kg on day −3), low-dose TBI (200 cGy on day −1) followed by living-donor kidneys transplant (day 0). The patients received donor bone marrow after living-donor kidneys were transplanted. Regarding stem cell transplantation, a population of so-called facilitating cells from donor bone marrow cells was enriched and used, after which point, Cy was administered at a dose of 50 mg/kg, 2 days later. Facilitating cells are identified as CD8-positive and αβγδ T-cell receptor-negative donor bone-marrow-derived cells that promote allogeneic stem cell reconstitution. In the recent report, two patients developed GVHD, and two patients experienced renal allograft losses. Although not yet a perfect induction of tolerance, the ability to discontinue immunosuppressive drugs in two-thirds of patients can be considered a great achievement. Combined hematopoietic stem cell and kidney transplantations have shown efficacy and safety, as well as validated the proof of principle of inducing tolerance by PTCy (64–67).

CONCLUSION

In this report, we outlined the history of Cy-induced immune tolerance. Collectively, clinical data suggest that the PTCy method for the induction of immune tolerance has been successfully translated from basic studies to clinical application. So far, the data have been adequately encouraging us to further develop PTCy approach in future studies.

AUTHOR CONTRIBUTIONS

KK, AT, KA, and ME wrote the manuscript and created the figures. All authors critically reviewed the manuscript and read and approved the final version of the manuscript.

REFERENCES

1. Ash RC, Horowitz MM, Gale RP, van Bekkum DW, Casper JT, Gordon-Smith EC, et al. Bone marrow transplantation from related donors other than HLA-identical siblings: effect of T cell depletion. *Bone Marrow Transplant.* (1991) 7:443–52.
2. Szydlo R, Goldman JM, Klein JP, Gale RP, Ash RC, Bach FH, et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *J Clin Oncol.* (1997) 15:1767–77. doi: 10.1200/JCO.1997.15.5.1767
3. Lo DJ, Kaplan B, Kirk AD. Biomarkers for kidney transplant rejection. *Nat Rev Nephrol.* (2014) 10:215–25. doi: 10.1038/nrneph.2013.281
4. Mayumi H, Umesue M, Nomoto K. Cyclophosphamide-induced immunological tolerance: an overview. *Immunobiology.* (1996) 195:129–39. doi: 10.1016/S0171-2985(96)80033-7
5. Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood.* (2001) 98:3456–64. doi: 10.1182/blood.V98.12.3456
6. Kappler JW, Staerz U, White J, Marrack PC. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature.* (1988) 332:35–40. doi: 10.1038/332035a0
7. MacDonald HR, Schneider R, Lees RK, Howe RC, Acha-Orbea H, Festenstein H, et al. T-cell receptor V beta use predicts reactivity and tolerance to Mlsa-encoded antigens. *Nature.* (1988) 332:40–5. doi: 10.1038/332040a0
8. Hengartner H, Odermatt B, Schneider R, Schreyer M, Walle G, MacDonald HR, et al. Deletion of self-reactive T cells before entry into the thymus medulla. *Nature.* (1988) 336:388–90. doi: 10.1038/336388a0
9. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature.* (1984) 307:168–70. doi: 10.1038/307168a0
10. MacDonald HR, Pedrazzini T, Schneider R, Louis JA, Zinkernagel RM, Hengartner H. Intrathymic elimination of Mlsa-reactive (V beta 6+) cells during neonatal tolerance induction to Mlsa-encoded antigens. *J Exp Med.* (1988) 167:2005–10. doi: 10.1084/jem.167.6.2005
11. Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. *Lancet.* (1992) 339:1579–82. doi: 10.1016/0140-6736(92)91840-5
12. Starzl TE, Demetris AJ, Trucco M, Ricordi C, Ildstad S, Terasaki PI, et al. Chimerism after liver transplantation for type IV glycogen storage disease and type 1 Gaucher's disease. *N Engl J Med.* (1993) 328:745–9. doi: 10.1056/NEJM199303183281101
13. Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med.* (2008) 358:353–61. doi: 10.1056/NEJMoa071074
14. Kawai T, Sachs DH, Sykes M, Cosimi AB. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med.* (2013) 368:1850–2. doi: 10.1056/NEJMc1213779
15. Stockman GD, Heim LR, South MA, Trentin JJ. Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. *J Immunol.* (1973) 110:277–82.
16. Berenbaum MC, Brown IN. Prolongation of homograft survival in mice with single doses of cyclophosphamide. *Nature.* (1963) 200:84. doi: 10.1038/200084a0
17. Berenbaum MC, Brown IN. Dose-response relationships for agents inhibiting the immune response. *Immunology.* (1964) 7:65–71.
18. Santos GW, Owens AH. Production of graft-versus-host disease in the rat and its treatment with cytotoxic agents. *Nature.* (1966) 210:139–40. doi: 10.1038/210139a0
19. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Kanakry CG. Optimized timing of post-transplantation cyclophosphamide in mhc-haploidentical murine hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* (2020) 26:230–41. doi: 10.1016/j.bbmt.2019.09.030
20. Sutton WT, Van H, Griffith BH, Preston FW. Drug effects on survival of homografts of skins. *Arch Surg.* (1963) 87:840–3. doi: 10.1001/archsurg.1963.01310170126021
21. Santos GW, Owens AH Jr. A comparison of the effects of selected cytotoxic agents on allogeneic skin graft survival in rats. *Bull Johns Hopkins Hosp.* (1965) 116:327–40.
22. Nomoto K, Eto M, Yanaga K, Nishimura Y, Maeda T. Interference with cyclophosphamide-induced skin allograft tolerance by cyclosporin A. *J Immunol.* (1992) 149:2668–74.
23. Shin T, Mayumi H, Himeno K, Sanui H, Nomoto K. Drug-induced tolerance to allografts in mice. I. Difference between tumor and skin grafts. *Transplantation.* (1984) 37:580–4. doi: 10.1097/00007890-198406000-00011
24. Mayumi H, Himeno K, Shin T, Nomoto K. Drug-induced tolerance to allografts in mice. VI. Tolerance induction in H-2-haplotype-identical strain combinations in mice. *Transplantation.* (1985) 40:188–94. doi: 10.1097/00007890-198508000-00016
25. Mayumi H, Himeno K, Shin T, Nomoto K. Drug-induced tolerance to allografts in mice. IV. Mechanisms and kinetics of cyclophosphamide-induced tolerance. *Transplantation.* (1985) 39:209–15. doi: 10.1097/00007890-198502000-00019
26. Mayumi H, Himeno K, Tokuda N, Nomoto K. Drug-induced tolerance to allografts in mice. VII. Optimal protocol and mechanism of

cyclophosphamide-induced tolerance in an H-2 haplotype-identical strain combination. *Transplant Proc.* (1986) 18:363–9.
27. Mayumi H, Good RA. Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. *J Exp Med.* (1989) 169:213–38. doi: 10.1084/jem.169.1.213
28. Colson YL, Wren SM, Schuchert MJ, Patrene KD, Johnson PC, Boggs SS, et al. A nonlethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *J Immunol.* (1995) 155:4179–88.
29. Dodd OJ, Ganguly S, Vulic A, Panoskaltsis-Mortari A, McDyer JF, Luznik L. Induction of major histocompatibility complex-mismatched mouse lung allograft acceptance with combined donor bone marrow: lung transplant using a 12-hour nonmyeloablative conditioning regimen. *Transplantation.* (2016) 100:e140–6. doi: 10.1097/TP.0000000000001480
30. Mayumi H, Tokunaga K. Cyclophosphamide-induced chimera-type tolerance to allografts: an overview of drug-induced immunological tolerance. *Fukuoka Igaku Zasshi.* (1990) 81:20–39.
31. Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med.* (1999) 5:1303–7. doi: 10.1038/15260
32. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nomoto K. Intrathymic clonal deletion of V beta 6+ T cells in cyclophosphamide-induced tolerance to H-2-compatible, Mls-disparate antigens. *J Exp Med.* (1990) 171:97–113. doi: 10.1084/jem.171.1.97
33. MacDonald HR, Hengartner H, Pedrazzini T. Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment. *Nature.* (1988) 335:174–6. doi: 10.1038/335174a0
34. Takeuchi A, Eto M, Yamada H, Tatsugami K, Naito S, Yoshikai Y. A reduction of recipient regulatory T cells by cyclophosphamide contributes to an anti-tumor effect of nonmyeloablative allogeneic stem cell transplantation in mice. *Int J Cancer.* (2012) 130:365–76. doi: 10.1002/ijc.26009
35. Ganguly S, Ross DB, Panoskaltsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, et al. Donor CD4+ Foxp3+ regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. *Blood.* (2014) 124:2131–41. doi: 10.1182/blood-2013-10-525873
36. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. *J Clin Invest.* (2019) 129:2357–73. doi: 10.1172/JCI124218
37. Kanakry CG, Ganguly S, Zahurak M, Bolanos-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. *Sci Transl Med.* (2013) 5:211ra157. doi: 10.1126/scitranslmed.3006960
38. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Nomoto K. Sequential mechanisms of cyclophosphamide-induced skin allograft tolerance including the intrathymic clonal deletion followed by late breakdown of the clonal deletion. *J Immunol.* (1990) 145:1303–10.
39. Tomita Y, Mayumi H, Eto M, Nomoto K. Importance of suppressor T cells in cyclophosphamide-induced tolerance to the non-H-2-encoded alloantigens. Is mixed chimerism really required in maintaining a skin allograft tolerance? *J Immunol.* (1990) 144:463–73.
40. Kong YY, Eto M, Omoto K, Umesue M, Hashimoto A, Nomoto K. Regulatory T cells in maintenance and reversal of peripheral tolerance *in vivo*. *J Immunol.* (1996) 157:5284–9.
41. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Maeda T, et al. Specific destruction of host-reactive mature T cells of donor origin prevents graft-versus-host disease in cyclophosphamide-induced tolerant mice. *J Immunol.* (1991) 146:1402–9.
42. Tomita Y, Nishimura Y, Harada N, Eto M, Ayukawa K, Yoshikai Y, et al. Evidence for involvement of clonal anergy in MHC class I and class II disparate skin allograft tolerance after the termination of intrathymic clonal deletion. *J Immunol.* (1990) 145:4026–36.
43. Maeda T, Eto M, Nishimura Y, Nomoto K, Kong YY. Role of peripheral hemopoietic chimerism in achieving donor-specific tolerance in adult mice. *J Immunol.* (1993) 150:753–62.
44. Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Host-reactive CD8+ memory stem cells in graft-versus-host disease. *Nat Med.* (2005) 11:1299–305. doi: 10.1038/nm1326
45. Kato K, Cui S, Kuick R, Mineishi S, Hexner E, Ferrara JL, et al. Identification of stem cell transcriptional programs normally expressed in embryonic and neural stem cells in alloreactive CD8+ T cells mediating graft-versus-host disease. *Biol Blood Marrow Transplant.* (2010) 16:751–71. doi: 10.1016/j.bbmt.2010.01.012
46. Gattinoni L, Zhong XS, Palmer DC, Ji Y, Hinrichs CS, Yu Z, et al. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat Med.* (2009) 15:808–13. doi: 10.1038/nm.1982
47. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. *Nat Med.* (2011) 17:1290–7. doi: 10.1038/nm.2446
48. Cieri N, Oliveira G, Greco R, Forcato M, Taccioli C, Cianciotti B, et al. Generation of human memory stem T cells after haploidentical T-replete hematopoietic stem cell transplantation. *Blood.* (2015) 125:2865–74. doi: 10.1182/blood-2014-11-608539
49. Roberto A, Castagna L, Zanon V, Bramanti S, Crocchiolo R, McLaren JE, et al. Role of naive-derived T memory stem cells in T-cell reconstitution following allogeneic transplantation. *Blood.* (2015) 125:2855–64. doi: 10.1182/blood-2014-11-608406
50. Luznik L, Engstrom LW, Iannone R, Fuchs EJ. Posttransplantation cyclophosphamide facilitates engraftment of major histocompatibility complex-identical allogeneic marrow in mice conditioned with low-dose total body irradiation. *Biol Blood Marrow Transplant.* (2002) 8:131–8. doi: 10.1053/bbmt.2002.v8.pm11939602
51. O'Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, Phelps M, et al. Nonmyeloablative bone marrow transplantation from partially HLA-mismatched related donors using posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2002) 8:377–86. doi: 10.1053/bbmt.2002.v8.pm12171484
52. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
53. Dezern AE, Luznik L, Fuchs EJ, Jones RJ, Brodsky RA. Post-transplantation cyclophosphamide for GVHD prophylaxis in severe aplastic anemia. *Bone Marrow Transplant.* (2011) 46:1012–3. doi: 10.1038/bmt.2010.213
54. Kanakry CG, Fuchs EJ, Luznik L. Modern approaches to HLA-haploidentical blood or marrow transplantation. *Nat Rev Clin Oncol.* (2016) 13:10–24. doi: 10.1038/nrclinonc.2015.128
55. Sugita J, Kawashima N, Fujisaki T, Kakihana K, Ota S, Matsuo K, et al. HLA-haploidentical peripheral blood stem cell transplantation with post-transplant cyclophosphamide after busulfan-containing reduced-intensity conditioning. *Biol Blood Marrow Transplant.* (2015) 21:1646–52. doi: 10.1016/j.bbmt.2015.06.008
56. Sugita J, Kagaya Y, Miyamoto T, Shibasaki Y, Nagafuji K, Ota S, et al. Myeloablative and reduced-intensity conditioning in HLA-haploidentical peripheral blood stem cell transplantation using post-transplant cyclophosphamide. *Bone Marrow Transplant.* (2019) 54:432–41. doi: 10.1038/s41409-018-0279-1
57. Kanakry CG, Tsai HL, Bolanos-Meade J, Smith BD, Gojo I, Kanakry JA, et al. Single-agent GVHD prophylaxis with posttransplantation cyclophosphamide after myeloablative, HLA-matched BMT for AML, ALL, and MDS. *Blood.* (2014) 124:3817–27. doi: 10.1182/blood-2014-07-587477
58. Kanakry CG, O'Donnell PV, Furlong T, de Lima MJ, Wei W, Medeot M, et al. Multi-institutional study of post-transplantation cyclophosphamide as single-agent graft-versus-host disease prophylaxis after allogeneic bone marrow transplantation using myeloablative busulfan and fludarabine conditioning. *J Clin Oncol.* (2014) 32:3497–505. doi: 10.1200/JCO.2013.54.0625
59. McCurdy SR, Kasamon YL, Kanakry CG, Bolanos-Meade J, Tsai HL, Showel MM, et al. Comparable composite endpoints after HLA-matched and HLA-haploidentical transplantation with post-transplantation cyclophosphamide. *Haematologica.* (2017) 102:391–400. doi: 10.3324/haematol.2016.144139

60. Chhabra AY, Leventhal J, Merchak AR, Ildstad S. HSCT-based approaches for tolerance induction in renal transplant. *Transplantation.* (2017) 101:2682–90. doi: 10.1097/TP.0000000000001837
61. Strober S. Use of hematopoietic cell transplants to achieve tolerance in patients with solid organ transplants. *Blood.* (2016) 127:1539–43. doi: 10.1182/blood-2015-12-685107
62. Oura T, Cosimi AB, Kawai T. Chimerism-based tolerance in organ transplantation: preclinical and clinical studies. *Clin Exp Immunol.* (2017) 189:190–6. doi: 10.1111/cei.12969
63. Leventhal JR, Elliott MJ, Yolcu ES, Bozulic LD, Tollerud DJ, Mathew JM, et al. Immune reconstitution/immunocompetence in recipients of kidney plus hematopoietic stem/facilitating cell transplants. *Transplantation.* (2015) 99:288–98. doi: 10.1097/TP.0000000000000605
64. Eto M, Nishimura Y, Matsuo K, Omoto K, Goto K, Kumuzawa J, et al. Prolongation of kidney graft survival by cyclophosphamide-induced tolerance in rats. *J Urol.* (1995) 153:1693–6. doi: 10.1016/S0022-5347(01)67506-2
65. Eto M, Takeuchi A. Current status and future perspective of tolerance induction in patients with kidney transplantation. *Fukuoka Igaku Zasshi.* (2016) 107:63–71. doi: 10.15017/1669231
66. Takeuchi A, Kato K, Akashi K, Eto M. Cyclophosphamide-induced tolerance in kidney transplantation avoids long-term immunosuppressive therapy. *Int J Urol.* (2018) 25:112–20. doi: 10.1111/iju.13474
67. Chen YB, Elias N, Heher E, McCune JS, Collier K, Li S, et al. Haploidentical hematopoietic cell and kidney transplantation for hematological malignancies and end-stage renal failure. *Blood.* (2019) 134:211–5. doi: 10.1182/blood.2019000775

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REVIEW
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Key Aspects of the Immunobiology of Haploidentical Hematopoietic Cell Transplantation

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Hematopoietic stem cell transplantation from a haploidentical donor is increasingly used and has become a standard donor option for patients lacking an appropriately matched sibling or unrelated donor. Historically, prohibitive immunological barriers resulting from the high degree of HLA-mismatch included graft-vs.-host disease (GVHD) and graft failure. These were overcome with increasingly sophisticated strategies to manipulate the sensitive balance between donor and recipient immune cells. Three different approaches are currently in clinical use: (a) *ex vivo* T-cell depletion resulting in grafts with defined immune cell content (b) extensive immunosuppression with a T-cell replete graft consisting of G-CSF primed bone marrow and PBSC (GIAC) (c) T-cell replete grafts with post-transplant cyclophosphamide (PTCy). Intriguing studies have recently elucidated the immunologic mechanisms by which PTCy prevents GVHD. Each approach uniquely affects post-transplant immune reconstitution which is critical for the control of post-transplant infections and relapse. NK-cells play a key role in haplo-HCT since they do not mediate GVHD but can successfully mediate a graft-vs.-leukemia effect. This effect is in part regulated by KIR receptors that inhibit NK cell cytotoxic function when binding to the appropriate HLA-class I ligands. In the context of an HLA-class I mismatch in haplo-HCT, lack of inhibition can contribute to NK-cell alloreactivity leading to enhanced anti-leukemic effect. Emerging work reveals immune evasion phenomena such as copy-neutral loss of heterozygosity of the incompatible HLA alleles as one of the major mechanisms of relapse. Relapse and infectious complications remain the leading causes impacting overall survival and are central to scientific advances seeking to improve haplo-HCT. Given that haploidentical donors can typically be readily approached to collect additional stem- or immune cells for the recipient, haplo-HCT represents a unique platform for cell- and immune-based therapies aimed at further reducing relapse and infections. The rapid advancements in our understanding of the immunobiology of haplo-HCT are therefore poised to lead to iterative innovations resulting in further improvement of outcomes with this compelling transplant modality.

Keywords: immunobiology, haploidentical, stem cell transplantation, NK-cells, graft-vs.-leukemia

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) remains a curative approach for many patients with malignant and non-malignant hematologic indications (1). However, timely availability of a suitable HLA-matched sibling donor (MSD) or adequately HLA-matched unrelated donor (MUD) remains a significant challenge in providing access to HCT. The likelihood of finding an optimal donor varies significantly among racial and ethnic groups with the chances of finding an appropriate donor ranging from 75% for whites of European descent to 16% for blacks of South or Central American descent (2). Although most candidates for HCT will have a donor or cord blood unit considered suitable (HLA-matched or minimally mismatched), even single allele mismatches negatively impact patient outcomes after HCT (3). Additionally, proceeding with an unrelated donor is a time- and cost-consuming process that can result in delay or suboptimal timing of HCT.

In contrast, haploidentical donors are available for >95% of patients in need of HCT (4). Biological children, parents, siblings, and frequently more distant family members who share one haplotype potentially qualify as donors (**Figure 1**). They can be readily identified and are typically available and motivated to donate bone marrow (BM) or peripheral blood stem cells (PBSC) to their family member in a timely fashion. This is particularly beneficial when unexpected events delay or expedite the need for HCT. Moreover, haploidentical donors can readily be tested in situations where there is concern for an underlying familiar predisposition syndrome and are typically available for a repeat stem cell collection, donor lymphocyte infusion or other cell therapeutic approaches which may be indicated if post-transplant complications such as graft failure, relapse, or infectious complications arise. Finally, if the selected family member had a poor stem cell mobilization for a PBSC graft or the optimal graft composition was not achieved then a different family member can be approached to serve as a haploidentical donor.

Historically, haploidentical HCT (haplo-HCT) was associated with high rates of graft vs. host disease (GVHD) and graft failure (5–7). With the introduction of efficient T-cell depletion (TCD) of the graft (8), haplo-HCT became feasible from a GVHD perspective. However, TCD led to an imbalance between host and donor T cells resulting in high rates of graft failure. This imbalance was overcome with the use of T-cell depleted "megadose" stem cell grafts (9, 10). Since then, nuanced *ex vivo* approaches to optimize the immunological composition

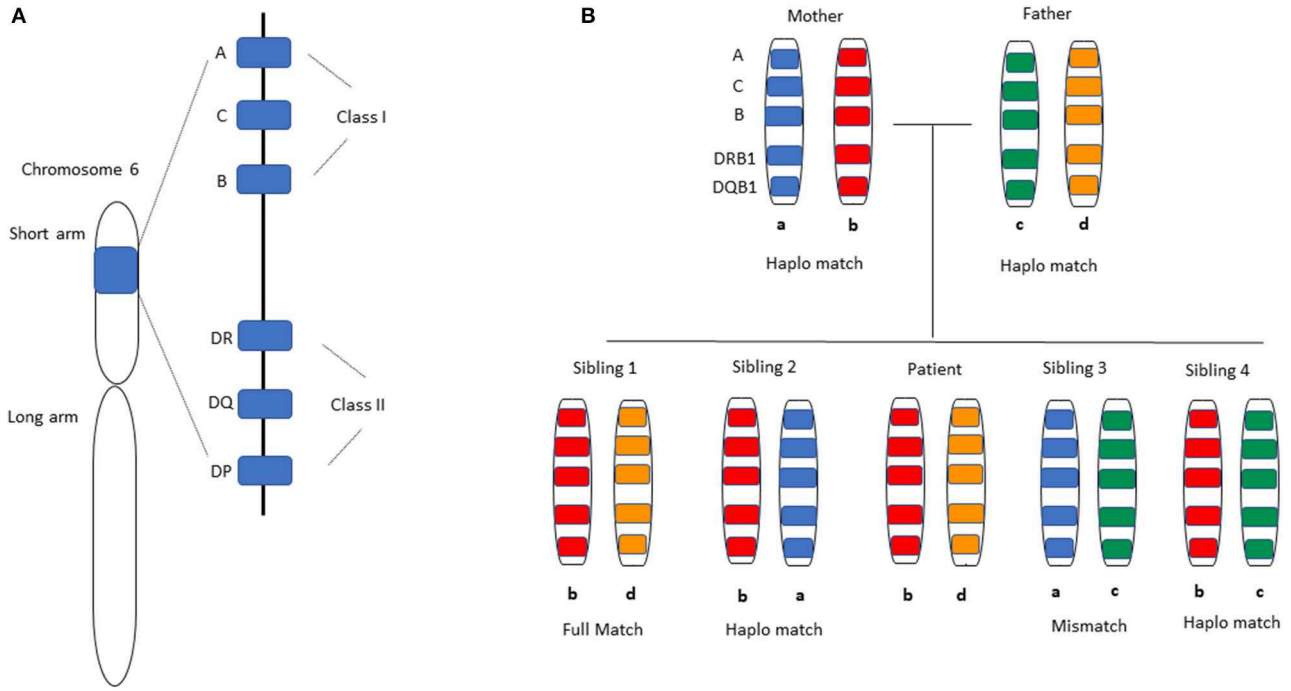

FIGURE 1 | HLA-matching in Haplo-HCT. **(A)** Distribution of HLA alleles on chromosome 6. All HLA alleles exist on the short arm of the chromosome, specifically 6p21.3. The classical HLA classification system is used clinically for matching donors and recipients in the transplant setting. HLA class I alleles -A, -B, and -C are expressed on all nucleated cells and display antigen to $CD8^+$ T-cells, while HLA Class II alleles -DR, -DQ, -DP are expressed on antigen-presenting cells and initiate a response by $CD4^+$ T-cells. Not shown are the non-classical HLA Class I alleles -E, -F, -G, -H, -J that are also present on the same chromosome arm. **(B)** A representative inheritance pattern of HLA alleles is demonstrated. For a patient with HLA allele distribution b and d as shown in the middle, each sibling has a 25% chance of being a full match based on inheritance of the same maternal (b) and paternal (d) alleles as the patient. Each sibling has a 50% chance of being a haploidentical match by virtue of having inherited one identical allele (b) from the parents. The likelihood of having inherited neither of the parental alleles that were inherited by the patient is 25% (complete HLA-mismatch).

of haploidentical grafts have been developed as outlined in this review.

A major milestone in promoting the wide-spread use and cost-efficient accessibility of haplo-HCT, including in resource-poor countries, was reached with the use of high-dose post-transplant cyclophosphamide (PTCy) to achieve *in vivo* attenuation of T cell alloreactivity (11). A different strategy using Granulocyte-colony stimulating factor (G-CSF) mobilized bone marrow grafts with extensive immunosuppression has been similarly feasible (12). In addition, a special emphasis is being placed on using natural killer (NK) cells to harness both innate and adaptive immunity in haplo-HCT. NK cells are uniquely regulated by activating and inhibitory receptors and can mediate a critical graft-vs.-leukemia (GVL) effect, also referred to as NK-cell alloreactivity, without mediating GVHD (13–15).

These approaches have contributed to a surge in the use of haplo-HCT in recent years (16). Furthermore, dramatic advances in the field of adoptive immune cell transfer have been applied to the haplo-HCT platform whereby donors could be readily approached for additional cell collections to enhance immunity against infections and relapse (17, 18). As haplo-HCT evolves to refine and establish its role in the field of transplantation, it is critical to examine the immunobiological properties unique to haplo-HCT and the effect of *ex vivo* or *in vivo* graft manipulation on the immunological content and trajectory of immune reconstitution.

CHALLENGES OF THE HLA-BARRIER IN HAPLO-HCT

Early trials of T-cell-replete haplo-HCT were associated with poor outcomes due to a high incidence of GVHD and graft rejection, resulting in ∼10% long-term survival (5–7, 19, 20). In the setting of grafting across a haploidentical HLA barrier, ∼2% of donor T cells mediate alloreactive reactions resulting in GVHD while residual host T cells mount host-vs.-graft responses leading to graft rejection (21–23). The ability to overcome the problem of GVHD despite the large HLA-disparity in haplo-HCT was first demonstrated by Reisner and colleagues with the successful transplantation of children with severe combined immunodeficiency (SCID) using T-cell depleted haploidentical grafts which differed at three major HLA loci (8). However, when this approach was extended to other indications in which a patient's underlying immune system is generally functional, the minimal T-cell content in the graft resulted in unopposed host-vs.-graft rejections and a high rate of graft failure. The latter was mediated by recipient anti-donor T lymphocyte precursors that survived the conditioning regimen (22, 24, 25), as well as anti-donor HLA antibodies (26) (**Figure 2**).

A second breakthrough that paved the way toward the broad application of haplo-HCT was the use of "megadose" grafts, targeting the infusion of a stem cell product containing on the order of $\geq 10 \times 10^6$/kg $CD34^+$ hematopoietic stem cells

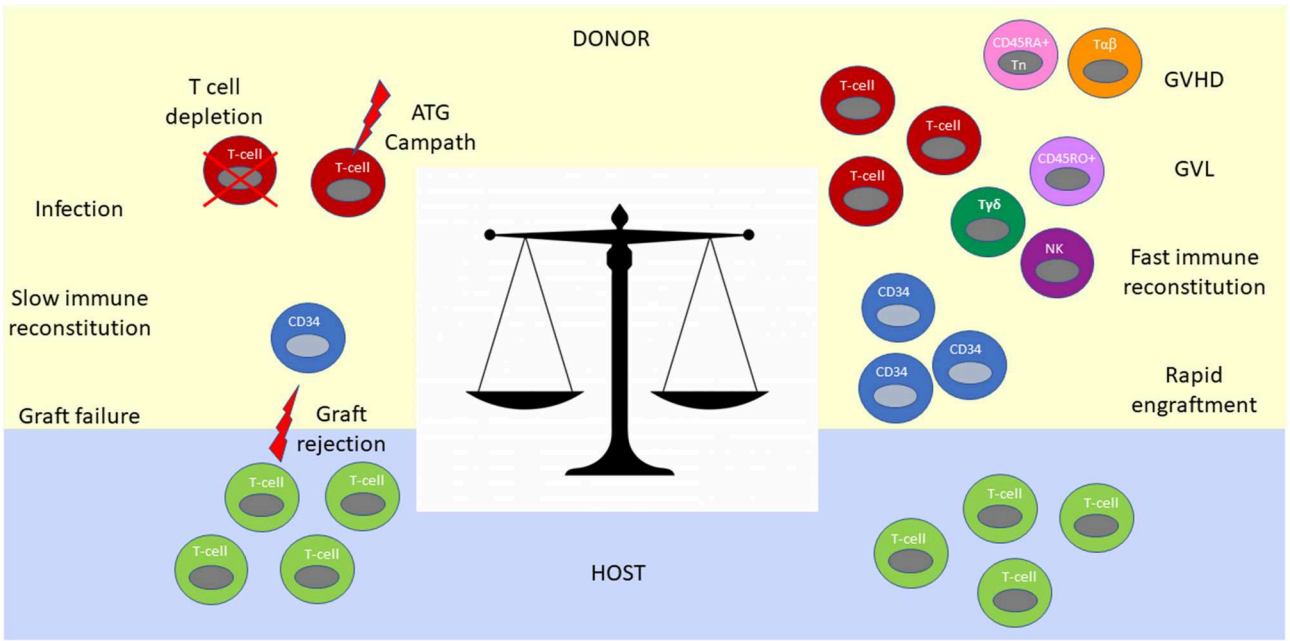

FIGURE 2 | Immunological balance determines outcomes after haplo-HCT. The graft contains $CD34^+$ and $CD34^-$ hematopoietic cells. $CD34^+$ progenitor and stem cells are required for engraftment and reconstitution of the bone marrow after transplantation into the host. T cells in the graft facilitate neutrophil engraftment, immune reconstitution, post-transplant infectious immunity and exert GVL effect **(Right)**. However, without an *ex vivo* (T cell depletion or CD34 positive selection) or *in vivo* (ATG or Campath) T cell depletion strategy, they mediate prohibitively severe GVHD **(Right)**. In contrast, extensive T cell depletion from the graft results in an immunologic imbalance between residual host and donor T cells favoring graft rejection **(Left)**. Extensive T cell depletion of the graft also results in slow immune constitution, infections and poor GVL control. To achieve an optimal immunologic balance, novel graft manipulation approaches selectively deplete T cells involved in GVHD ($CD45RA^+$ T cell and αβ- T cell depletion strategies), while maintaining beneficial immune cells such as NK cells and γδT cells in the graft.

while retaining the threshold dose of $\leq 4 \times 10^4$/kg T cells established in the SCID patients (9, 10, 27, 28). The underlying immunologic effect of megadose grafting was attributed to tolerance induction of host anti-donor cytotoxic T cell precursors by donor $CD34^+$ stem cells or by $CD34^+$ derived regulatory immune cells endowed with a "veto"-effect in a TNFα mediated mechanism (29, 30). Intensified myeloablative conditioning (MAC) with 8 Gy total body irradiation (TBI), thiotepa, rabbit anti-thymocyte globulin (ATG) and fludarabine (replacing cyclophosphamide after 1995) (31) to eliminate host T cells, followed by G-CSF mobilized megadose T-cell depleted PBSC grafts (initially using soybean agglutination and erythrocyte resetting and later immunomagnetic selection of $CD34^+$ HSCs) without any additional post-transplant immunosuppression was refined over the years (28, 32). This approach ultimately demonstrated primary engraftment in 95% of patients with acute leukemia ($n = 104$), with 6 of 7 patients who initially experienced graft failure engrafting successfully after second transplantation. Although acute and chronic GVHD were largely prevented, a significant non-relapse mortality (NRM) of 36.5% was observed largely owing to post-transplant infections (27 of the 38 patients died of infectious complications) and substantial relapse risk. The 2-year event-free survival (EFS) probability among patients receiving transplantation in any complete remission (CR) was 47%, while the EFS for those transplanted in relapse was 4% (27).

Despite the tremendous advances toward clinical feasibility of haplo-HCT, these early studies embodied the challenge of achieving a sensitive immunologic balance during transplantation across haploidentical HLA-barriers. This challenge is reflective of the need for extensive T-cell depletion and immunosuppression to control GVHD on the one hand, and facilitation of engraftment, immune reconstitution, protection from infections, and prevention of relapse on the other (**Figure 2**). This conundrum has fueled the iterative improvement of modulating immunity in the context of haplo-HCT as outlined below.

CURRENT HAPLO-HCT PLATFORMS

In vivo Haplo-HCT Strategies With Unmanipulated Stem Cell Grafts

Post-transplantation Cyclophosphamide (PTCy)

Post-transplantation high-dose cyclophosphamide (PTCy), when administered in a specific time-frame after graft infusion, efficiently attenuates alloreactive T cells from both donor and host and prevents GVHD and graft rejection. This immunological effect of PTCy was first observed in the 1960s in animal models of allogeneic skin grafts whereby cyclophosphamide administration within a window of up to 4 days after grafting delayed rejection (33). Subsequent preclinical studies defined the role of PTCy in the setting of allogeneic HCT and showed the benefits of its use with respect to engraftment and GVHD (34–36). Importantly the concurrent immunosuppression of T cells with cyclosporine or steroids interfered with PTCy-tolerogenic effects (37, 38), indicating that high proliferative rates are critical for the PTCy immunomodulatory mechanism (39).

Initial mechanistic studies based on murine skin allografting models attributed the PTCy-effect to the selective depletion of alloreactive T cells. Based on these hypotheses, the presumed depletion was dependent on the heightened cytotoxic sensitivity of newly primed and highly proliferative alloreactive T cells (particularly $CD4^+$ T cells) at the peak of anti-host and anti-donor T cell expansion, aided by a favorable balance between effector T cells and regulator T cells (Tregs) as well as an additional intrathymic clonal deletion of alloreactive T cell precursors (40–44). Suppressive immune cells were only felt to have an adjunct role in maintaining tolerance (45, 46). However, recent work by Kanakry and colleagues formally tested the putative immunologic mechanisms (selective destruction of alloreactive T cells, intrathymic clonal deletion of alloreactive T cells and induction of suppressor T cells) in dedicated murine PTCy haplo-HCT models (47). These studies suggest that PTCy reduces $CD4^+$ T cell proliferation but does not eliminate alloreactive T cells and instead functionally impairs the T-cell response to alloantigens and induces the rapid and preferential recovery and expansion of regulatory T cells (Treg). Treg resistance to PTCy is based on their differential expression of aldehyde dehydrogenase (ALDH) (48). Evidence for the importance of the role of Tregs after PTCy is exemplified by the development of severe and fatal GVHD in the context of $Foxp3^+$ Treg depletion, as well as additional data showing that Tregs are required for PTCy-mediated protection against GVHD (49). Studies in thymectomized mice also suggested the dispensability of the thymus in this process (47). Advances in this active field of preclinical and clinical study are poised to further elucidate and facilitate adaption of the PTCy platform for different clinical scenarios. Increasing experience with this platform and the potential for PTCy-mediated bi-directional tolerance induction also lends itself to further exploration of this approach in the setting of combined solid organ and bone marrow transplantation (44).

The first clinical study of unmanipulated haplo-HCT with PTCy was conducted in the setting of non-myeloablative (NMA) conditioning with administration of PTCy at 50 mg/kg on day +3 and an added immunosuppressive regimen of mycophenolate mofetil (MMF) and tacrolimus starting on day +4 in 13 patients (50) (**Figures 3A**, **4C**). Subsequent prospective clinical trials, administering PTCy either on day +3 or on days +3 and +4, demonstrated rates of graft failure and GVHD comparable to those reported with reduced intensity conditioning (RIC) HLA-matched sibling and MUD HCTs with a trend toward a lower risk of extensive chronic GVHD among recipients of two doses of PTCy (50). These studies paved the way for the increased investigation and clinical use of haplo-HCT with PTCy (**Figure 3B**).

GIAC Approach (G-CSF-Mobilization, Intensified Post-transplant Immunosuppression, ATG and Combination of PBSC and BM Allografts)

The GIAC approach using T-cell replete haploidentical grafts was pioneered at Peking University (12, 51). This approach uses ATG

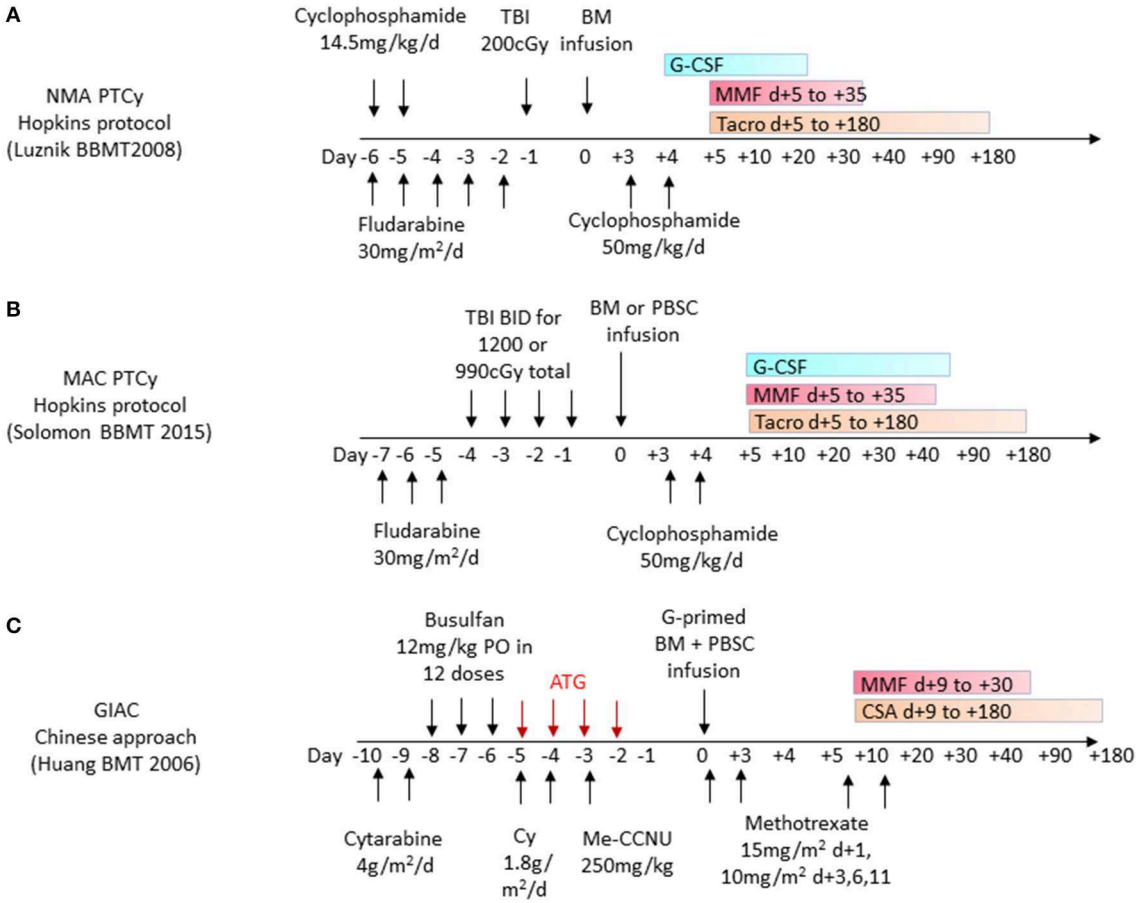

FIGURE 3 | Frequently used haplo-HCT regimens. **(A)** Non-myeloablative (NMA) conditioning with administration of post-transplant cyclophosphamide (PTCy) as part of the Hopkins protocol for haplo-related donor HCT uses cyclophosphamide 50 mg/kg/day on days +3 and +4 and additional GVHD prophylaxis with oral MMF and tacrolimus (Tacro) starting on day +5. **(B)** Myeloablative conditioning (MAC) protocol with administration of post-transplant cyclophosphamide 50 mg/kg/day given on days +3 and +4 and additional GVHD prophylaxis with oral MMF and tacrolimus starting on day +5. **(C)** GIAC haplo-HCT protocol using a combination of G-CSF primed bone marrow (BM) and peripheral blood stem cells (PBSC) administered after a conditioning regimen including ATG on days −5 to −2. GVHD prophylaxis includes short-course Methotrexate in addition to MMF and cyclosporine (CSA).

as part of the conditioning regimen, which affects recipient T cells and facilitates engraftment. Owing to its long half-life, it also exerts effects on donor T cells and therefore impacts GVHD and post-transplant immunity. The graft consists of a combination of G-CSF-primed bone marrow and PBSC, thereby combining the advantages of both elements. PBSC grafts contain 2–3-fold higher $CD34^+$ cells and a log-fold higher T cell dose than are typically contained in a steady-state bone marrow graft (52), and this has been shown to accelerate engraftment and decrease the relapse rate (**Figures 3C**, **4B**).

The higher T cell dose in PBSC grafts adversely affects chronic GVHD but not acute GVHD rates in unrelated donor HCT (53). Multiple mechanisms may contribute to why acute GVHD rates are not drastically higher despite the high T cell dose. These include preferential dendritic cell mobilization and T cell polarization (54, 55), attenuating effects on costimulatory molecules such as CD86 on APCs and CD28 on $CD4^+$ T cells (56, 57), as well as IL-10 mediated T-cell suppression by monocytes (58). Several studies underscored the benefit of utilizing G-CSF mobilized bone marrow, leading to less acute and chronic GVHD while maintaining engraftment rates comparable to PBSC (59) and have attributed these effects to differences in cytokine milieu, T-cell polarization and T-cell hyporesponsiveness (60–62).

In the initial study of 171 patients using GIAC, most of whom had ALL, AML, or CML, all patients engrafted with sustained full donor chimerism. The rates of leukemia-free survival and incidences of grade II-IV acute GVHD and extensive chronic GVHD were comparable to MUD HCT (12, 53). A prospective multicenter study of AML patients has demonstrated that transplant outcomes with the GIAC strategy have also been comparable to MSD HCT (63). Although a modified approach using G-CSF primed haploidentical bone marrow and extensive GVHD prophylaxis has also been applied in Europe (64), the GIAC strategy has been used most extensively in China and therefore patients transplanted with this strategy represent a large cohort of haploidentical transplants HCT treated to date (65).

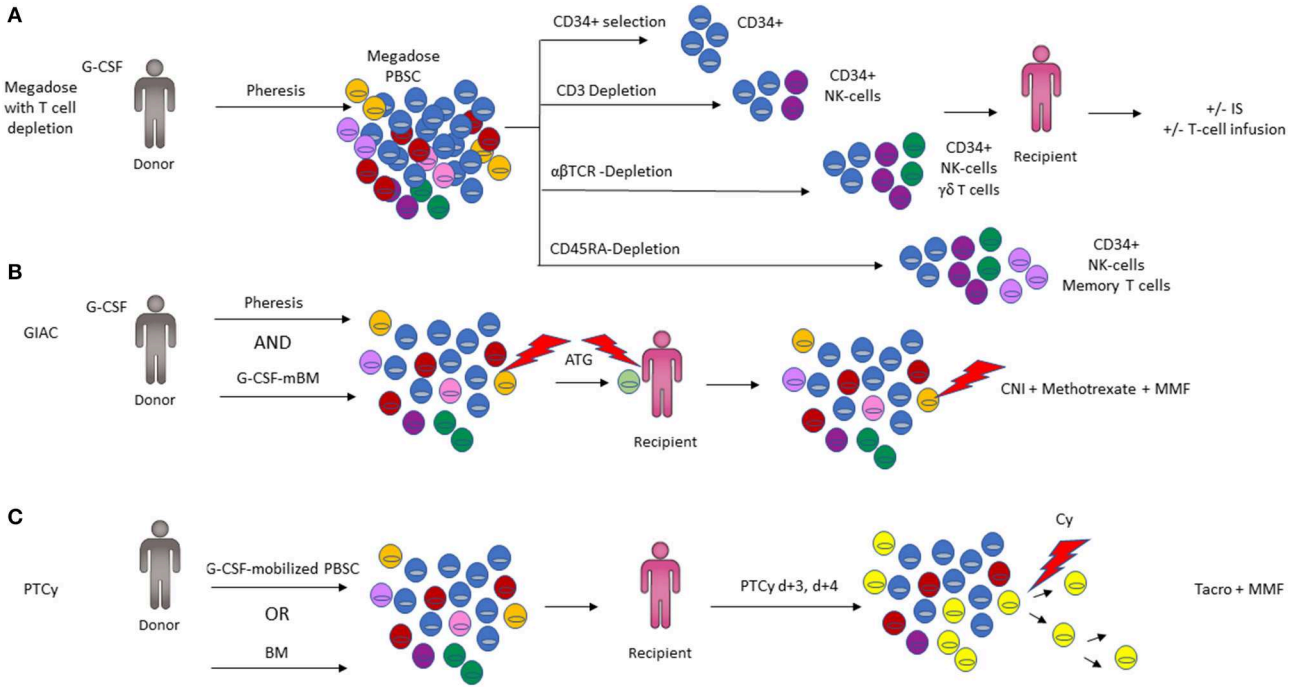

FIGURE 4 | Comparison of the three major haplo-HCT platforms. **(A)** Four different *ex vivo* T-cell depletion protocols are shown, with the resulting cell composition in the graft. CD34-positive selection preferentially isolates the hematopoietic stem and progenitor fraction required for engraftment with minimal immune cell content (top panel). Depletion of $CD3^+$ T-cells results in a graft composed predominantly of $CD34^+$ and NK cells (2nd panel). Depletion of αβ-T cells depletes T cells involved in mediating GVHD but retains beneficial immune cells such as NK cells and γδ- T cells in the graft. CD45RA-Depletion removes naïve T cells including cells responsible for alloreactivity and GVHD, while retaining memory T cells including cells vital for immunity against infections (3rd panel). Additional immunosuppression (IS) and/or infusion of T-cell subsets may be employed post-transplant to optimize engraftment (3rd panel). **(B)** Representation of the GIAC protocol indicating a G-CSF primed bone marrow (BM) and peripheral blood stem cell (PBSC) graft, with ATG targeting T-cells derived from both the donor and recipient. GVHD prophylaxis with methotrexate, a calcineurin inhibitor (CNI), and MMF targets residual T cells (3rd panel). **(C)** Post-transplant cyclophosphamide (PTCy) functionally impairs actively proliferating recipient and graft derived T cells while favoring Treg recovery (color of cells corresponds to **Figure 2**; Tregs are depicted in bright yellow).

Haploidentical Hct With *ex vivo* T Cell Depletion or Anergy Induction Strategies

$CD34^+$ Cell Selection

The establishment of procedures for the *ex vivo* removal of T cells from the graft in the late 1970s by Reisner, O'Reilly and colleagues, represented a tremendous breakthrough toward the feasibility of utilizing haploidentical donors. In the initial approach, T cells were eliminated from the bone marrow by first rosetting with sheep red blood cells followed by differential soybean agglutination of residual T lymphocytes in the non-rosetting population. This yielded an un-agglutinated fraction containing a high proportion of colony-forming cells without any detectable T cell alloreactivity, and abrogated lethal GVHD in murine models (66, 67). This strategy was applied in the first clinically successful haploidentical HCT of an infant with AML, leading to sustained hematopoietic engraftment without GVHD until relapse occurred 11 weeks after HCT (68). Three infants with SCID were also treated with this approach of whom 2 had sustained engraftment and none developed GVHD (8).

$CD34^+$ selection, now in wide-spread use in TCD transplants, was first introduced in the 1990s. This process utilizes a $CD34^+$ directed antibody coupled to immunomagnetic beads to positively select $CD34^+$ cells and isolate them over a magnetic column. This effectively eliminates all other immune cells, including T-, B-, NK-cells, dendritic cells and monocytes from the graft (69, 70). This process was further refined with the use of micromagnetic beads, which had the advantages of high purity selection via attachment to single cells and safe infusion into patients (71). Aversa and colleagues of the Perugia group pioneered a novel haploidentical HCT platform incorporating an intensified conditioning regimen to eliminate host T cells and administering megadose T cell depleted grafts without additional post-grafting immunosuppression (28, 72). Handgretinger et al. tested this approach with G-CSF mobilized megadose PBSC grafts in 39 children lacking suitable donors and observed low rates of GVHD, but significant relapse and treatment-related mortality (TRM) (73). Investigators from Perugia further evaluated this system in adults with high-risk leukemia using megadose haplo-HCT, demonstrating 91% primary engraftment and low rates of GVHD without post-transplant GVHD prophylaxis (27) (**Figure 4A**, top panel).

$CD3^+$ Cell Depletion

To improve post-transplant immune reconstitution, control of infections and prevention of relapse, further iterations of immunomagnetic graft engineering were developed (74). This

included the elimination of $CD3^+$ T cells and $CD19^+$ B cells using a negative immunomagnetic selection method to deplete these subsets from the graft. Stem cells, NK cells, myeloid precursors, monocytes, and other progenitor cells important for engraftment are preserved (75). This strategy maintains innate immunity in the graft while removing $CD3^+$ T cells capable of inducing GVHD. Depletion of $CD19^+$ B cells was introduced to reduce the risk of post-transplant lymphoproliferative disease (PTLD) (73) and GVHD (76). While the depletion of donor B-cells reduces the risk of PTLD, it does not address PTLD arising from residual host B cells. Instead, this can be addressed with the inclusion of rituximab or Campath (but not the T cell directed agents ATG or OKT3) into the conditioning regimen (77, 78). Several centers established $CD3^+/CD19^+$ depletion as a feasible approach for patients lacking a suitable donor, with excellent primary engraftment and reduced rates of GVHD correlating with the remaining $CD3^+$ cell/kg content of the graft. However, the low OS rate of 31% remains primarily attributable to infections and relapse, suggesting that further improvement of TCD haplo-HCT is needed (79, 80) (**Figure 4A**, second panel).

αβT-Cell/B-Cell Depletion

With emerging recognition of γδT cells (81), a yet more sophisticated approach was developed for GVHD prevention. In contrast to αβ-T-cell receptor (TCR) expressing T cells, γδ-TCR expressing T-cells are not implicated in mediating GVHD (82) but do exhibit important functions characteristic of innate immune recognition and anti-tumor effects (83, 84). These cells represent 1–20% of all $CD3^+$ circulating T lymphocytes in human peripheral blood and the majority of resident T cells in skin and mucosa. Their TCR heterodimer consists of a γ and δ chain encoded by a limited repertoire of V, D, and J gene segments. The two major Vδ1 and Vδ2 subsets are distinguished based on their TCRδ composition. Whereas, $V\delta1^+$ cells are typically associated with a Vγ1/2/3/5/8 chain, the majority of $V\delta2^+$ T cells express an invariant TCR harboring Vγ9. The Vγ9δ2 TCR is expressed by the majority of peripheral γδ T cells, whereas γδ T cells including other Vδ elements are predominantly enriched at epithelial surfaces and the skin (81, 84). Analogous to NK cell biology, γδT cells are fine-tuned by activating and inhibitory receptors and recognize conserved non-peptide antigens that signal potential danger or cellular stress. The activating receptor NKG2D is broadly expressed in γδT cells and functions synergistically with the γδ-TCR as a costimulatory receptor (85, 86).

γδT cells have heterogenous functions, ranging from protection against intra- and extracellular pathogens or malignant cells to modulation of the immune response and tissue homeostasis. They contribute to pathogen clearance through the production of granulysin, defensins, and cytotoxic effector molecules such as perforin and granzymes (84). γδT cells secrete proinflammatory cytokines involved in protective immunity against viruses, intracellular pathogens (TNF-α and IFN-γ), extracellular bacteria, fungi (IL-17), and extracellular parasites (IL4, IL5, IL13), and have been shown to exhibit lytic activities against leukemia, lymphoma and carcinoma cells (87–89). Indeed, increased γδT-cell numbers after allogeneic HCT were associated with a lower incidence of infections and improved disease-free survival (DFS) in several studies (90–92).

In a pediatric trial using αβ-T cell/B-cell depleted haplo-HCT, γδ-T cells were the predominant T-cell population in the initial weeks after transplantation, specifically expanded in response to CMV reactivation, and displayed cytotoxicity and degranulation when challenged with primary leukemia blasts *in vitro* (93). These effects were increased after exposure to zoledronic acid, suggesting that the anti-leukemic capacity of γδ-T cells could further be enhanced (94). Outcomes with the αβ-T cell/B-cell depleted haplo-HCT approach in which no additional GVHD prophylaxis was employed appear promising both in children with malignant (95) and non-malignant conditions (96), and when compared with MUD and MMUD HCTs in a retrospective analysis of children transplanted for acute leukemias (97). However, the high incidence of viral infections reported by some groups highlights the potential to further improve *ex vivo* T-cell depletion strategies (98) (**Figure 4A**, third panel).

CD45RA-Depletion

As our understanding of T cell differentiation status and phenotype has become increasingly sophisticated, so have approaches to tailor graft composition further (99, 100). αβ-T cells exist as distinct subsets that can be differentiated by cell surface phenotype: naïve (T_N), stem cell memory (T_{SCM}), effector (T_E), effector memory (T_{EM}), and central memory (T_{CM}). The $CD45RA^+CD62L^+$ T_N subset is antigen inexperienced, has a more diverse TCR repertoire than memory T cells and clonally expands following T cell priming to execute short-lived effector functions. They ultimately differentiate into memory subsets, which is associated with downregulation of CD45RA and upregulation of CD45RO. Studies in mouse models demonstrated that T_N mediated severe GVHD, whereas T_{CM} induced milder GVHD and T_{EM} were devoid of GVH activity (101–105). Importantly memory T cells transferred infectious immunity and GVL activity in these models (106).

Based on the premise that elimination of T_N from the graft could significantly reduce GVHD while maintaining pathogen- and tumor-specific immunity, Bleakley and colleagues developed a novel graft-engineering strategy using immunomagnetic beads coupled to a monoclonal Ab targeting CD45RA. The latter antigen is expressed on all T_N, but absent on Treg, T_{CM} and most T_M (107). This strategy was initially studied in patients with high risk hematologic malignancies undergoing MSD HCT, utilizing a 2-step selection procedure with a $CD34^+$ selection of stem cells (a minor subset of which expresses CD45RA) followed by depletion of $CD45RA^+$ cells from the $CD34^-$ fraction. This study demonstrated engraftment in all patients ($n = 35$), prompt immune recovery without excessive rates of infection or relapse and low chronic GVHD, but interestingly no reduction in acute GVHD although the latter was readily steroid-responsive (108).

Clinical results with CD45RA-depletion in the context of haplo-HCT are so far limited. A study of 17 pediatric patients with high risk hematologic malignancies using a RIC conditioning with total lymphoid irradiation (TLI) but without TBI or serotherapy, administered a $CD34^+$ selected PBSC product on day 0, followed by a CD45RA-depleted PBSC product

which had been collected the following day, and ultimately a donor NK cell product administered on day +6 with the use of Sirolimus or MMF post-transplant. Rapid neutrophil engraftment and memory T-cell reconstitution was observed, without any infectious deaths and with 76.5% of patients alive at a median of 225 days after HCT. Grade III-IV acute GVHD and chronic GVHD were seen in 3 and 6 of 17 patients, respectively (109). In a second small study, 5 children with combined immunodeficiency and chronic viral infections received a combination of a $CD34^+$ selected product and the CD45RA-depleted fraction of the CD34-negative product with post-HCT prophylaxis consisting of Cyclosporine and MMF. One patient died with graft failure. In the 4 engrafted patients, viral infections cleared within 2 months after HCT and an early T cell response against viral pathogens was documented in 2 patients (110). Further studies will be needed to further define the role of this approach in haplo-HCT (**Figure 4A**, bottom panel).

Ex vivo Induction of T Cell Anergy With CTLA-4Ig

An early strategy to minimize T-cell alloreactivity by interfering with the priming of alloreactive T cells in haplo-HCT was explored in a pediatric trial. This involved collection of patients' peripheral blood mononuclear cells (PBMC) prior to the start of myeloablation and a 36-h *in vitro* incubation of the recipient cells with non-mobilized donor bone marrow in a mixed lymphocyte reaction (MLR) setting in the presence of CTLA-4Ig, a fusion protein which inhibits priming of alloreactive T cells by inhibiting costimulatory signaling between the B7 protein family (CD80/CD86) on APCs and CD28 on T cells (111). This reduced the frequency of T cells recognizing alloantigens of the recipient while preserving responsiveness to alloantigens of other persons. In this trial of 11 evaluable patients most of which had persistent disease at the time of HCT, 5 were alive and in CR at 4.5–29 months after transplant with 3 patients developing steroid-responsive acute GVHD of the gut only. There were no deaths attributable to GVHD (112). However, this approach has not been explored further.

Photodynamic Purging of Adoptive T Cell Therapy Following TCD Haplo-Hct

A different approach to augment the TCD graft with an adoptive T cell therapy product devoid of alloreactive T cells is a process termed photoallodepletion. Prior to G-CSF mobilization of the PBSC graft, donors undergo non-mobilized leukapheresis to obtain T cells. Donor T cells are then incubated with recipient PBMC in an MLR in the presence of TH9402, a photosensitizer similar to rhodamine. T cell activation in the MLR, which occurs selectively in the alloreactive T cells but spares Tregs and pathogen-specific T cells, is associated with P-glycoprotein pump inhibition leading to mitochondrial accumulation of TH9402 in alloreactive T cells (113, 114). Subsequent activation of TH9402 with visible light leads is then selectively toxic to and eliminates alloreactive T cells via an oxidative damage mechanism (115). Early results from a clinical trial in which patients received the photodynamically allodepleted T-cell product subsequent to a $CD34^+$ selected graft appear promising (116).

ROLE OF INNATE IMMUNITY IN HAPLO-HCT

NK-cells are an important component of the innate immune system providing protection against infectious pathogens and cancer. Recent studies have elucidated that human NK cell diversity is much broader than the traditional distinction via $CD56^{bright}$ and $CD56^{dim}$ subsets reflective of differentiation stage and cytotoxic potential. The ability of NK cells to differentiate into long-lived cells with memory capacity (117) and the discovery of non-NK innate lymphoid cells has highlighted the complexity and potential roles of innate immune cells after HCT (118, 119). NK cells have potent anti-leukemia effector capacity, respond to viral infections via release of toxic granules, and facilitate engraftment without mediating GVHD. This is particularly important in the setting of heavily T cell-depleted grafts or T-cell directed post-transplant immunosuppression and has inspired a rich field of investigation to augment NK cell immunity in the context of HCT to develop leukemia-directed NK-cell based cellular therapies.

NK-cell activity is governed by the balance of a system of activating and inhibitory NK cell receptors (120). Activating signals are provided by receptors such as NKG2D, CD94/NKG2C and Natural Cytotoxicity Receptor (NCRs) including NKp30, 44, and 46 and by activating killer-cell Ig-like receptors (KIR). NKG2D recognizes MHC-class I related stress-ligands that can be upregulated by tissues in response to infection, inflammation, DNA-damage, and malignant transformation (121), while CD94/NKG2C binds to the non-classical HLA-E molecules and senses overall HLA-Class I expression on cells (**Figure 5A**). NK cells utilize a unique process to balance tolerance to self under steady state conditions with the ability to mediate an immune response to pathogens or malignant cells. This is referred to as NK-cell education or licensing (122), is in large part regulated by inhibitory KIR receptors and impacts NK-cell alloreactivity in the setting of haplo-HCT and allogeneic NK-cell therapies (123).

KIRs are either activating or inhibitory based on their structure. The KIR nomenclature incorporates the number of extracellular Ig-like domains (two in KIR2D vs. three in KIR3D) and whether the KIR contains a long or short tail (KIR2DL vs. KIR2DS). KIRs are further numbered in order of their discovery within their structural group (KIR2DL1 vs. KIR2DL2). KIRs with long tails are generally inhibitory (with exception of KIR2DL4) and KIRs with short tails function as activating receptors according to presence or absence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (124). There is tremendous variability within the KIR repertoire owing to a high degree of polymorphism among individual KIR genes as well as their organization and recombination within haplotypes (**Figures 5B,C**) (125). An individual's genetic KIR repertoire is determined by the inherited composition of centromeric and telomeric A and B haplotypes (**Figure 5B**). Group A haplotypes contain fewer genes and predominantly those encoding for inhibitory KIRs. Additionally, the activating KIR2DS4 gene exist as an inactive deletion variant, termed KIR1D in the majority of Caucasians, leaving the framework gene KIR2DL4 as the

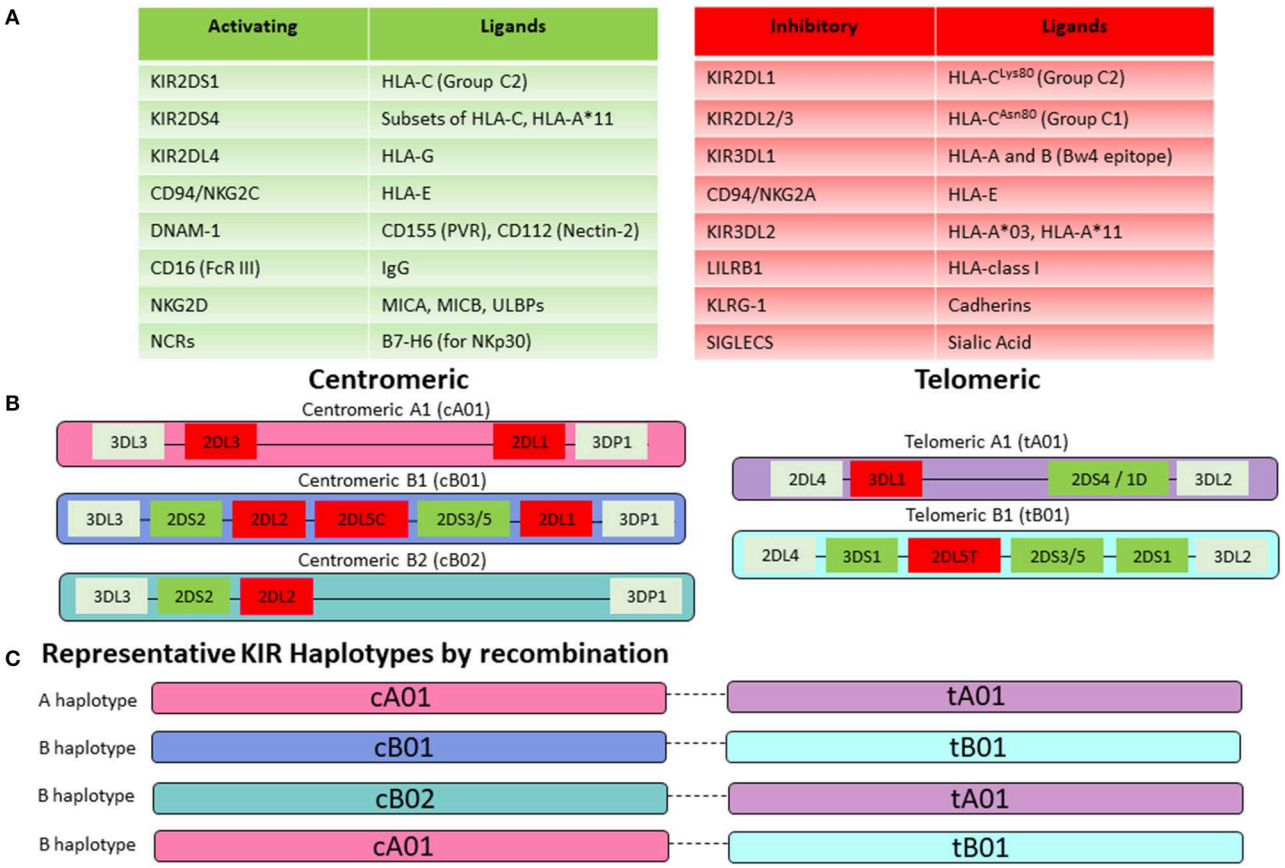

FIGURE 5 | NK-cell receptor repertoire. **(A)** NK-cell activity is mediated by a balance of activating and inhibitory signaling. Key activating receptors and their corresponding ligands are listed in the green table, while inhibitory receptors are displayed in the red table. **(B)** KIR genes are highly polymorphic and organized in centromeric and telomeric motifs with structural variation that creates multiple gene content haplotypes. Group A haplotype motifs are characterized by fewer genes and predominantly those encoding for inhibitory KIRs. In contrast, Group B haplotype motifs are enriched for activating KIRs. **(C)** Centromeric and telomeric motifs are paired together to generate either a KIR A haplotype (composed of centromeric and telomeric A motifs) or a KIR B haplotype (containing at least one centromeric or telomeric B motif). Representative KIR haplotypes by recombination are shown here. Prominent linkage disequilibrium has been noted within the centromeric and telomeric motifs but not between them, suggesting that pairing occurs by recombination between the centromeric and telomeric regions.

sole receptor on this haplotype with any activating function (126, 127). In contrast, Group B haplotypes are enriched for activating KIRs. Two groups of KIR haplotype can be assigned based on the combination of the centromeric and telomeric motifs. Presence of a centromeric or telomeric B-haplotype constitutes a KIR B haplotype whereas the combination of a centromeric and a telomeric A-haplotype results in a KIR A haplotype (**Figure 5C**). Although more than 50 different haplotypes have been described, there are 11 common haplotypes derived by reciprocal recombination, which collectively account for 94% of Caucasian haplotypes examined by Jiang et al. (128). Distribution of a KIR gene in the centromeric or telomeric region of chromosome 19q13.4 is further thought to impact KIR-mediated regulation of NK-cell activity (129). Additionally, KIR-cell surface expression at the protein level may vary substantially from the inherited KIR gene profile. This is attributable to the fact that KIRs are stochastically expressed on NK cells and each NK cell may therefore display a different cell-surface profile of inhibitory or activating KIRs (130). For the most accurate prediction of NK-cell alloreactivity between haploidentical donor and recipient, KIR-genotyping alone is insufficient and determination of the KIR phenotype (by flow cytometry) should also be pursued.

The majority of inhibitory KIRs recognize classical (HLA- A, B, and C) or non-classical HLA-class I molecules (HLA-G) as their cognate ligands (**Figure 5A**) (131). KIR genes are located on chromosome 19 whereas HLA-genes are located on chromosome 6. KIR and HLA genes therefore segregate independently, and an individual may or may not express the cognate HLA-ligand for any given KIR. This forms the basis for the concept of "education" or "licensing" of NK-cells, which allows NK-cells to maintain self-tolerance under physiologic conditions, while retaining the ability to mount an immune response (132). When NK cells encounter the matching HLA-class I ligand for their inhibitory KIR (based on the requisite germline inheritance of the appropriate HLA and KIR genes and their expression patterns on

individual NK cells), they are considered "educated" or "licensed" and refrain from an attack on healthy tissues under steady state. However, when NK cells are accustomed to this inhibitory signal and subsequently encounter a cell that does not express the appropriate KIR-ligand ("missing ligand"), this situation renders them functional to mount an effector response, if the target also expresses stress-ligands that trigger activating NK-cell receptors (133). A missing ligand may be encountered on malignant cells due to HLA-class I downregulation, or HLA-mismatched allogeneic transplantation such as haplo-HCT, when the recipient does not express the corresponding HLA-ligand (**Figure 6**). NK-cells are considered "unlicensed" when they do not encounter the matching HLA-class I ligand for their given inhibitory KIR. Due to the lack of exposure to their corresponding ligand, unlicensed NK-cells are "un-educated" and hyporesponsive at steady state rather than being triggered by self-tissues lacking the ligand (134). Unlicensed NK cells require a higher threshold for activation. However, in the absence of KIR inhibition, they can mediate higher levels of effector function when they receive strong stimulatory signals under inflammatory conditions (such as CMV infection or in the posttransplant setting) or when triggered for antibody-dependent cellular cytotoxicity (ADCC) (135, 136). Given that NK cells may surface-express variable combinations and densities of inhibitory KIRs, NK-cell education occurs on a continuum along which individual NK cells display graded levels of responsiveness based on their KIR profile and engagement of cognate HLA-class I ligands (122, 137, 138).

Since the model of NK-cell alloreactivity in the context of mismatched HCT was first proposed, a number of studies have evaluated its clinical impact (139). For the interpretation of HCT studies evaluating the role of NK-cell alloreactivity it is critical to consider the definition of the KIR-mismatch model employed in each study (131, 140) (**Figure 6**). The "KIR ligand-ligand mismatch model" is based on the hypothesis that the presence of the corresponding HLA-ligand prevents NK-cell alloreactivity, whereas a missing ligand in the HCT recipient triggers NK cell alloreactivity. However, while this model accounts for HLA-class I mismatches, it does not consider KIR-genotype or phenotype. In contrast, the "KIR receptor-ligand mismatch model" accounts for the fact that a missing ligand is irrelevant if NK cells do

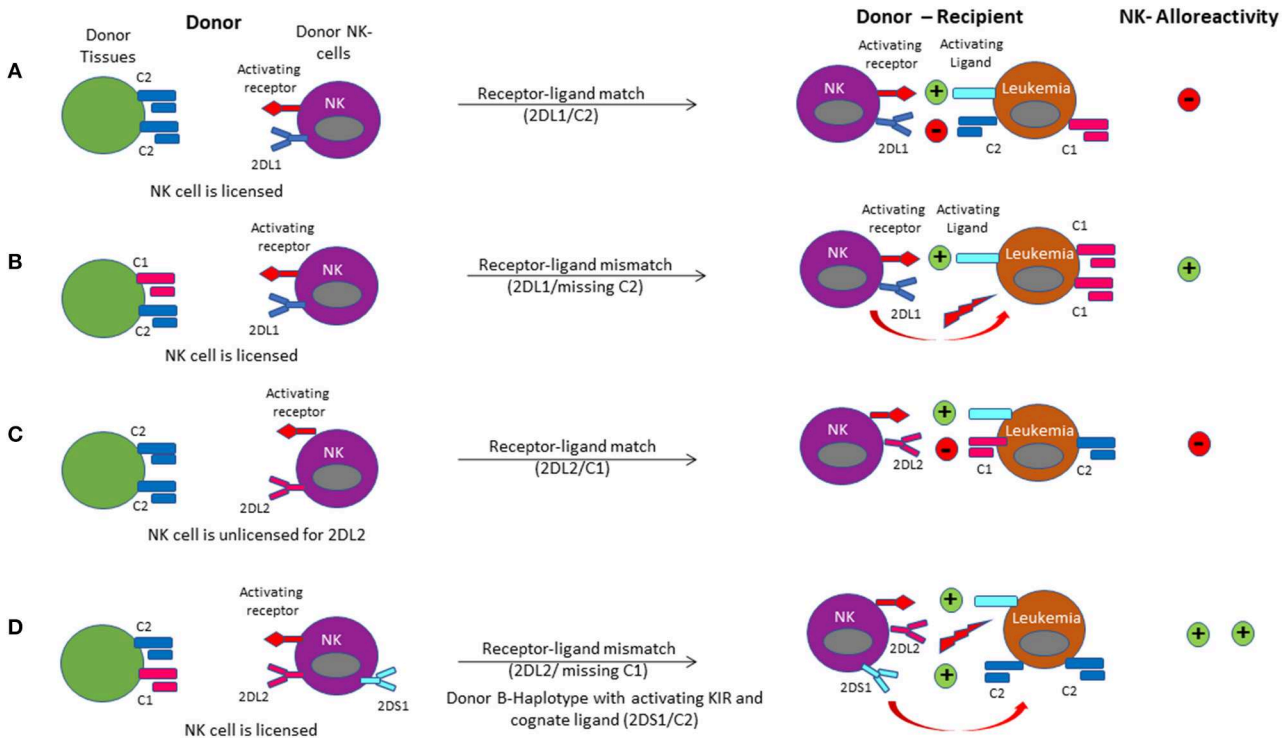

FIGURE 6 | NK cell alloreactivity in haplo-HCT is demonstrated via the different models of receptor-ligand mismatch. **(A)** The donor-derived NK cell is licensed when its KIR2DL1 receptor had been engaged by expression of its cognate C2 ligand in the donor environment. Upon infusion of the licensed NK cell into the recipient, a leukemia cell expressing the C2 ligand will not activate the NK cell due to a receptor-ligand match. **(B)** A receptor-ligand mismatch occurs when the donor-derived NK cell is licensed, but the recipient does not express the C2 ligand (missing ligand). Provided that it is further driven by stimulation through activating receptors, this results in activation of the licensed donor NK cell upon infusion into the recipient, leading to a graft-vs.-leukemia effect. **(C)** If the donor does not express the appropriate class I ligand for its KIR receptor (HLA and KIR segregate independently), the donor NK cell is unlicensed. In this case, donor NK cells are accustomed to a missing ligand. They may be activated when encountering strong activating signals (like activating cytokines) or be further inhibited when encountering the inhibitory ligand in the recipient. **(D)** Licensing of the NK cell for the C1 ligand occurs in the donor. Upon transplant into the host, the missing C1 ligand coupled with binding of the activating ligand with the activating receptor on the NK cell results in alloreactivity. Binding of the activating KIR receptor KIR2DS1 to the C2 ligand on the target leukemia cell enhances NK cell alloreactivity.

not express the corresponding KIR for a mismatched HLA-class I ligand. Therefore, this model incorporates the HLA-ligand repertoire in the recipient as well as the donor KIR genotype and ideally phenotype. Other groups have employed the "KIR-haplotype model" which takes into consideration the presence or absence of a B-KIR haplotype in the donor, as a measure of enrichment for activating vs. inhibitory KIRs. Use of this model demonstrated a reduced risk of leukemia relapse when patients were transplanted from donors with centromeric B-haplotypes (141–143). Similarly, more recent approaches have focused on the predicted overall degree of inhibitory and activating KIR-KIR ligand interactions between the recipient and potential donors with a highly variable KIR repertoire. This allows for selection of an optimal donor, even when the transplant recipient's HLA-class I repertoire is such that all KIR ligands are expressed and a missing-ligand scenario is unachievable.

Ruggeri et al. first established that a NK-cell alloreactivity of the donor toward recipient (based on KIR receptor-ligand mismatch in the GVL direction and presence of alloreactive clones against recipient targets) lowered the AML relapse risk in the context of *ex vivo* depleted haplo-HCT (72). These results were subsequently consolidated in a larger cohort of 112 AML patients, where transplantation from a NK-cell alloreactive donor was associated with a significantly lower relapse rate (3% compared to 47%) when transplanted in complete remission and better EFS when transplanted in relapse (34% compared to 6%) or CR (67% compared to 18%) (144). Subsequent studies of sibling donor, unrelated donor (URD), and umbilical cord blood (UCB) donor sources have yielded variable results (14). Some studies showed no benefit or even inferior survival resulting from a mismatch in the KIR/KIR-ligand system. This may be partly related to the variable definition of KIR-mismatch models and transplant regimens used. In contrast, a large analysis in AML patients undergoing 9/10 or 10/10 URD employed an algorithm to predict the strength of inhibition between the ubiquitous KIR3DL1 and its ligand HLA-B and found that combinations with absent of weak inhibition were associated with significantly lower rates of relapse and overall mortality (145). The extent of T-cell depletion may also play an important role, since the presence of T cells in the graft affects NK cell reconstitution leading to lower KIR-receptor expression (146). Lastly, given that a KIR ligand-ligand mismatch implies an absence of a KIR ligand in the host that is present in the donor, it equates with the presence of a major HLA-class I mismatch. It is therefore not unexpected that such mismatch leads to significant T-cell alloreactivity and poor survival unless T-cell reactivity is minimized with methods such as TCD.

A retrospective analysis of 161 patients receiving TCD haploidentical allografts confirmed a beneficial role of NK cell alloreactivity. In the presence of KIR-receptor-ligand mismatches in the GVL direction, expression of activating KIR2DS1 or KIR3DS1 was associated with a significant reduction in NRM, largely owing to 50% reduction in infection rates (147). While much of the benefits of NK cell alloreactivity are reported for myeloid indications, a pediatric study of 85 patients undergoing TCD haplo-HCT showed that patients transplanted for ALL from a KIR B-haplotype donor had a significantly better EFS than those with KIR haplotype A donors. Additionally, a higher KIR B-content score (based on the number of centromeric and telomeric KIR B motifs) was associated with a significant reduction in relapse risk (148). Although limited by use of a KIR ligand-ligand model, a study of haplo-HCT with PTCy for various hematologic malignancies found that KIR-ligand mismatch was associated with a lower incidence of relapse and better PFS for patients transplanted in relapse but had no significant impact on those transplanted in CR (149). A growing ability to navigate the complexities of the KIR-system, such as recognition of varied strengths of inhibition among subtypes of inhibitory KIRs and its ligands resulting in discrete hierarchies of anti-leukemic cytotoxicity will aid in further revealing how donor selection based on KIR-compatibility may improve outcomes (145). While the beneficial effects of NK-cell alloreactivity are mostly documented in the context of *ex vivo* T cell-depleted haplo-HCT, the growing adaptation of T-cell replete haplo-HCT affords the opportunity to carefully study the role of NK-cell alloreactivity in these platforms.

IMMUNE RECONSTITUTION AFTER HAPLO-HCT

Transplant outcomes are directly related to the achievement of an acceptable restoration of the immune system. Several cell subsets play a key role in the protection toward infections and disease recurrence. In general, innate immunity recovers early after transplant and represents the first line of defense against pathogens. Specifically, monocytes followed by neutrophils and NK cells arise in the first month after transplant. Adaptive immunity mediated by T and B cell lymphocytes recovers later and is crucial for both immune tolerance maintenance and long-term protection against infections and disease relapse. T cell reconstitution can occur through two different mechanisms: thymus-independent T cell peripheral expansion of infused donor memory T cells and thymus-dependent *de novo* generation of donor T cells from donor hematopoietic progenitors (150).

While the kinetics of immune reconstitution and its correlation with HCT outcomes are well-established in the setting of matched donor transplant, more studies are needed in the setting of haplo-HCT. Different donor sources do not represent the only cause of possible differences in immune reconstitution kinetics. Specific haplo-HCT platforms and GVHD prophylaxis approaches are also crucial factors to consider (151). As detailed above, two major haplo-platforms are currently used: T- cell replete haplo-HCT that use an *in vivo* T-cell depletion with ATG or PTCY, and TCD haplo-HCT in which the graft is *ex vivo* manipulated with a CD34-positive selection or a T-cell negative selection. Here, we review the immune reconstitution of different blood cell subsets after different types of haplo-HCT (**Figure 7**).

Monocytes

Monocytes are the first immune subset to recover after HCT. Rapid and robust monocyte $CD14^{+}$ cell reconstitution has been correlated with the improvement of transplant outcomes in

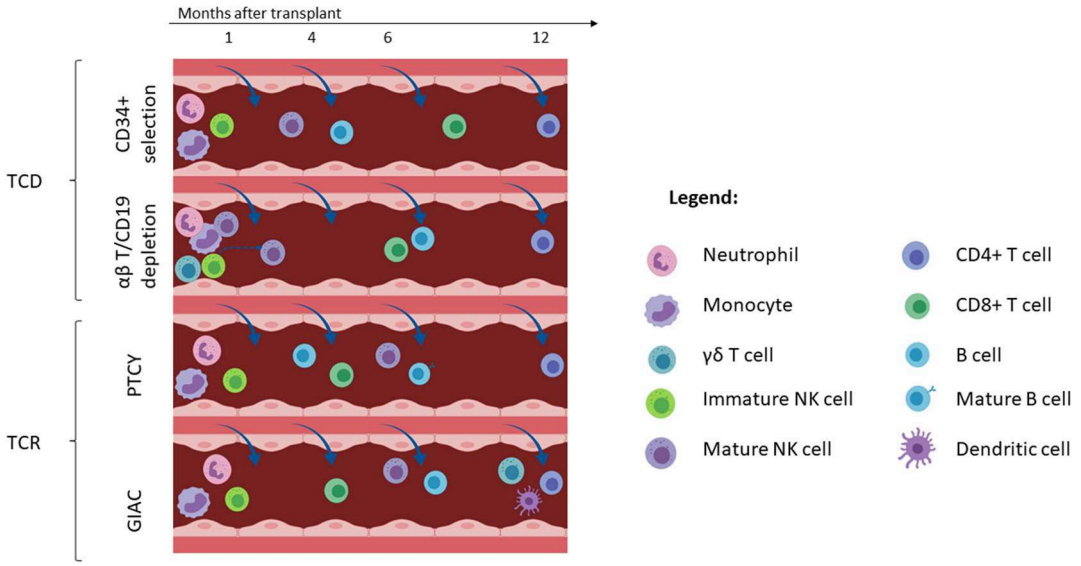

FIGURE 7 | Immune reconstitution with different haploidentical transplant platforms. Only subsets which have been characterized by published primary data for each platform are included in this figure (e.g., data on dendritic cell reconstitution has only been described for the GIAC approach). Cells are depicted at the approximate time-point of reaching the lower range of normal. T-cell depleted (TCD) haploidentical transplant is associated with early recovery of neutrophils, monocytes, immature NK cells and rapid NK cell maturation which takes 6–8 weeks (top panel). Additionally, αβ-T cell/CD19 depletion is associated with early detection of γδT cells and mature NK cells that are infused with the graft infusion (2nd panel from the top). B cell recovery is delayed with CD19 depletion relative to $CD34^+$ selection. T-cell replete (TCR) haploidentical transplant performed with post-transplant cyclophosphamide (PTCy) and GIAC protocols is associated with early reconstitution of immature NK cells (3rd panel from the top). It is also associated with earlier reconstitution of $CD8^+$ T-cells than TCD protocols. The GIAC protocol is associated with delayed dendritic cell recovery (bottom panel). This figure was created using BioRender.com.

the setting of MSD (152) and UCB-HCT (153). Recently, a study by Turcotte and colleagues showed that higher absolute monocyte count (AMC) and higher classic monocyte subsets ($CD14^{bright}$ $CD16^-$) at day +28 were associated with a reduced risk of relapse and TRM, better 2-yr OS, and improved 2-yr PFS in a cohort of patients transplanted for different hematological malignancies using both RIC or MAC regimens and different stem cell sources (154). AMC was influenced by the graft origin, with a higher AMC found in UCB but no differences between BM and PBSC. However, no haplo-HCTs were included in this study. In a separate cohort of 144 patients treated with MAC conditioning for hematological malignancies, receiving a T-cell replete graft consisting of G-CSF-mobilized BM and PBSC from HLA-haploidentical or MSDs, the monocytes recovered rapidly, and the AMC was above the normal range starting from the first month to the first year after transplant. Both patient groups received GVHD prophylaxis with Methotrexate, Tacrolimus, MMF, and Cyclosporine with the addition of ATG in the haploidentical group (GIAC protocol). Monocyte reconstitution was comparable between recipients after HLA-matched and haplo-HCT on days +30, 90, and 180 after transplantation. None of the patient transplant characteristics impacted monocyte recovery in the multivariable analysis (155). Finally, in a pediatric cohort of 40 patients receiving TCD haplo-HCT using CD34 positive selection or CD3/CD19 cell depletion, monocyte expansion was rapid, reaching normal values for age within 30 days of transplant. Moreover, no differences in monocyte recovery were seen between different graft purification and conditioning intensity regimens (156).

Neutrophils

Depending on the study, neutrophil engraftment is defined by the presence of more than 500 or 1,000 neutrophils/μL of blood and represents a crucial step in the early phase after transplant. Prolonged neutropenia is associated with severe infection and increased TRM (157). In the setting of a T-cell replete transplant, neutrophil recovery occurs quickly. With GIAC protocols, the median neutrophil engraftment was achieved at 14 days (range 9–25) (158, 159), whereas with the RIC PTCY platform using BM grafts and Tacrolimus and MMF GVHD-based prophylaxis, the median time to neutrophil recovery was 15 days (range 11–42) (11). For both protocols, patients received recombinant human granulocyte colony-stimulating factor (rhG-CSF) from day +6 or +4 to engraftment, respectively.

In the context of TCD HCT using the Perugia protocol with $CD34^+$ selected megadose grafts, the median time to neutrophil recovery was 11 days (range 9–30) without G-CSF support (27). Studies using CD3/CD19 cell depletion in adult patients also showed rapid neutrophil recovery, with a median time of 12 days (range 9–50) without the addition of G-CSF (79, 80). Similar results were seen in a cohort of pediatric patients with acute leukemia undergoing MAC transplant. Specifically, patients in the αβ-T cell-depleted haplo-HCT had a faster neutrophil recovery compared to MUD, mismatch unrelated donors (mMUD), and those treated with

Methotrexate and Calcineurin-inhibitors, with median time to neutrophil engraftment of 13 (range 6–23), 19 (range 9–46), and 20 days (range 10–120), respectively (97). All three groups received ATG during the conditioning for prevention of graft failure and GVHD, and none of the patients received G-CSF to accelerate neutrophil recovery. Taken together, these data show that haplo-HCT provides a comparable or even expedited neutrophil recovery compared to standard matched donor-HCT.

Dendritic Cells

Dendritic cells (DCs) represent a rare population in the peripheral blood, accounting for 0.15–0.7% of mononuclear cells (160). In the context of T-cell replete haplo-HCT using the GIAC protocol, Wang and colleagues measured the frequencies of DCs and their subsets among white blood cells (WBCs) after haplo-HCT, including $CD123^+$ plasmacytoid DCs (pDCs) and $CD11c^+$ myeloid DCs (mDCs). Recipients had strikingly decreased proportions of DCs (0.49% vs. 0.27%, $P = 0.025$), mDCs (0.27% vs. 0.14%, $P < 0.001$), and pDCs (0.04% vs. 0.02%, $P = 0.008$) in the WBC compartment at ∼180 days post-haplo-HCT compared to healthy subjects. Since, it was reported that primary human DCs were the most potent expander of the γδ T cell subset $V\delta2^+$ (161), the authors also investigated whether the recovery levels of $V\delta2^+$ T cells were associated with the DC content following transplantation. Bivariate correlation analysis showed that the proportion of mDCs, but not DCs and pDCs, in WBCs was significantly correlated with the recovery of $V\delta2^+$ T cells after haplo-HCT. Specifically, slow recovery of mDCs was associated with a slow recovery of $V\delta2^+$ T cells in this haplo-HCT setting (162).

Chang and colleagues also described a slower DCs recovery at +15 and 30 days after HCT compared to those in the HLA-matched recipients in another study (158). In their protocol, ATG was administrated only in the haplo-group. Indeed, it was described that ATG not only induced a tolerogenic phenotype in human DCs (163), but was also able to mediate a complement-mediated lysis of DCs (164). In summary, these findings may explain the delay in DC recovery in the setting of the haplo-HCT using the GIAC protocol. The kinetics of DC reconstitution in other haplo-HCT settings, remain to be fully characterized.

Natural Killer (NK) Cells

Due to the need to perform an extensive T cell depletion in haplo-HCT, anti-tumor efficacy is largely dependent on the graft-vs.-leukemia effect exerted by NK cells that eradicate residual leukemic blasts surviving the preparative regimen (72, 165–167). In the haplo-HCT setting performed through the infusion of positively selected $CD34^+$ cells, the first emergence of fully functioning, KIR alloreactive NK cells from hematopoietic progenitors may require at least 6–8 weeks, and therefore the benefit offered by their anti-leukemia effect is delayed (168–171). In the setting of αβ-T-cell/CD19 depletion, generation of NK cells from donor HSC takes ∼8 weeks but circulating NK cells can be detected earlier after transplant due to infusion with the graft (172). Moreover, CMV reactivation in this setting was associated with an expansion of memory-like NK cells ($NKG2C^+$, $CD57^+$, KIR^+) as early as 3 months after HCT (173). Surprisingly, in a pediatric comparison between TCD haplo-HCT performed with CD34 positive selection or CD3/19 negative selection, NK-cell recovery was faster in patients receiving PBSC from $CD34^+$ positive selection in the first 4 months after transplant (156).

In the T-cell replete haplo-HCT setting using PTCY, Russo and colleagues described that donor alloreactive NK cells infused with the graft were killed by cyclophosphamide (174). This translated into a delay of NK recovery and maturation resulting from a profound reduction after cyclophosphamide administration following a robust proliferation of donor-NK cells in the early phase after graft infusion. The absence of aldehyde dehydrogenase (ALDH)-positive NK cells suggested that they were susceptible to cyclophosphamide cytotoxicity, and this was then confirmed using an *in vitro* assay of mafosfamide-induced cell death (174). On the other hand, Russo et al. reported an IL-15 peak in patient sera at day +15 after transplant that was associated with a progressive increase of NK cells expressing an immature phenotype ($CD62L^+$, $NKG2A^+$, KIR^-) between day +15 and day +30 (174). The normal distribution of NK phenotypes was achieved only between 9 and 12 months after transplant, with a decrease of $CD56^{bright}$, NKG2A, and CD62L expression and an increase of maturation markers (CD16, CD57, and KIR). KIR expression returned to normal levels around day +60, but NKG2A expression decreased only after 6 months. Interestingly, in this cohort of patients, there was no difference in PFS between patients with or without a predicted KIR alloreactivity, suggesting that the protective anti-tumor activity of NK cells is dampened after T-cell replete haplo-HCT using the PTCY platform (174).

Another group described the transient and predominant expansion of an unconventional subset of NK cells characterized by a specific phenotype: $NKp46^{neg/low}$, $CD56^{dim}$, $CD16^{neg}$, $CD94/NKG2A^{high}$ starting from the second week after transplant and maintained until the 7th week (175). This unconventional population retained its proliferative capacity and the ability to differentiate into the $CD56^{bright}$ subsets ($NKp46^+$, $CD56^{bright}$, $CD16^-$ cells) in response to IL-15 and IL-18. Despite the unconventional NK cells expressing a high level of activating receptors (NKG2D and NKp30), Granzyme-B and Perforin, they displayed a defective *in vitro* cytotoxicity highlighting again the need to improve NK reconstitution after PTCy haplo-HCT (175). Similar results were reported in the GIAC protocol in which early and higher expression of CD94/NKG2A was inversely correlated with KIR expression, and was associated with worse survival (176). The same group showed that NK cells from patients who developed GVHD had a lower expression of NKG2A, lower proliferative capacity and an increased rate of apoptosis, but retained their cytotoxicity after *in vitro* co-culture with the K562 cell line (177).

Finally, in contrast to TCD haplo-HCT, KIR-mismatch analysis between donor-recipient pairs when using only HLA and KIR genotyping without consideration of the KIR phenotype, was unable to predict post-transplantation outcomes in multivariate analyses in the setting of haplo-HCT using the GIAC protocol (178). However, it has been reported that KIR-driven NK cell alloreactivity is better predicted if donor KIR genotype is considered in conjunction with KIR cell surface expression

(130). Moreover, in haplo-HCT using the GIAC protocol, the higher number of T-cells infused in the graft contributed to the high incidence of acute GVHD (178). This resulted in a need for increased immune suppression, thereby affecting NK alloreactivity.

T Cells

Achievement of an acceptable T cell reconstitution after HCT represents a crucial goal and correlates with better transplant outcomes. Impairment of T cell reconstitution is more pronounced after T cell depletion (152). In the context of T-cell replete haplo-HCT using the GIAC protocol, $CD3^+$ T cell counts were 125, 883, 1,163, and 1,308 cells/μL at 30, 90, 180, and 360 days after HCT, respectively (158). A lower median $CD3^+$ T cell count was reported after NMA haplo-HCT using a BM graft with PTCy, Tacrolimus and MMF based GVHD prophylaxis, with 206 cells/μL at day 40 and 219 cells/μL at day 100 (179). On the other hand, $CD3^+$ T-cell recovery was more rapid with 338 cells/uL at day +30 after MAC haplo-HCT using PBSC grafts with PTCy, MMF, and sirolimus GVHD-based prophylaxis (180).

In the setting of T-cell replete haplo-HCT with both GIAC and PTCy-based protocols, $CD8^+$ T cells recovered earlier than $CD4^+$ T cells (158, 181–183). Faster $CD8^+$ T cell recovery at day +90 correlated with higher $CD3^+$ cells in the graft but was not associated with a higher incidence of GVHD (184). The same studies highlighted that the recovery of $CD4^+$ T cells was impaired for the whole first year after transplant, but failed to demonstrate a correlation between delay in $CD4^+$ T cell reconstitution and NRM as was shown in the HLA-matched donor setting (185). Notably, in the GIAC experience the delay of $CD4^+$ T-cell reconstitution was compensated by the proportional increase of the $CD8^+$ T cell- and monocyte fractions, and the NRM was relatively low (19.5% in the haplo group vs. 17.4% for the matched-sibling donor cohort). This was likely due to patient care improvements, especially the management of CMV reactivation (158).

A retrospective EBMT registry study including both adult and pediatric patients undergoing haplo-HCT found an association between higher $CD3^+$, $CD4^+$, and $CD8^+$ T-cell counts and better OS with less NRM (186). However, in the multivariable analysis only higher $CD3^+$ and $CD8^+$ T-cell counts correlated with lower NRM. No association was found between any of the T-cell, B-cell, or NK-cell subset counts with relapse-related mortality. In this study, the majority of patients were treated with TCD haplo-HCT using both $CD34^+$ selection and CD3/19 depletion (186). In the context of αβ T-cell depleted haplo-HCT, $CD3^+$, and $CD3^+/CD8^+$ T-cell recovery was slower compared to MUD or MMUD-HCT until 6 months after transplant (97). Recovery of $CD4^+$ T cells was delayed only in the first 3 months and became even better at 1 year after haplo-HCT compared to MUD and MMUD. In this pediatric experience, haplo-HCT patients did not receive any additional pharmacological GVHD prophylaxis, whereas MUD and MUD HCT were performed using standard calcineurin-based GVHD prophylaxis and short-term methotrexate (97).

T memory stem cells (T_{SCM}) represent a subset of early-differentiated human memory T cells with stem cell-like properties. T_{SCM} and naïve T cells (T_N) both express naïve markers such as CD45RA, CCR7, and CD62L, but in distinction to T_N and similar to other memory subsets, T_{SCM} are characterized by CD95 expression. In the context of haplo-HCT using PTCy, two different groups elegantly showed that donor-derived T_{SCM} reconstitute early after transplant, representing the majority of both CD4 and CD8 T cells at day +8. At the polyclonal, antigen-specific, and clonal level, T_{SCM} lymphocytes were preferentially derived from differentiation of T_N infused within the graft, whereas most memory infused lymphocytes are purged by PTCy (182, 187).

Regulatory T (Treg) Cells

Treg cells play a key role in the modulation of immune tolerance after HCT. Higher Treg content in the graft has been associated with better OS and lower aGVHD (188), whereas a reduced frequency of Tregs contributed to cGVHD incidence after matched-donor transplant (189). In the matched donor setting, Kanakry and colleagues showed that Treg, especially memory CD45RA-Treg, were preserved and recovered rapidly while conventional T (Tcon) naïve cells were reduced when PTCy was used as the sole method of GVHD prophylaxis (48). This was ascribed to the high levels of aldehyde dehydrogenase (ALDH), as the major *in vivo* mechanism of Cyclophosphamide resistance in the Treg population. In addition, murine studies demonstrated the importance of Tregs for GVHD reduction in the context of the PTCy-based GVHD prophylaxis (49).

In the T-cell replete haplo-HCT setting using PTCy, naïve Tregs increased after cyclophosphamide administration. This was attributed to the lower Ki67 levels compared to the memory subsets at day +3. In addition, Tregs exhibited a lower proliferation profile compared to Tcons, suggesting a lower susceptibility to PTCy in the haploidentical setting (182). This effect seems to be enhanced when PTCy is combined with sirolimus instead of a calcineurin inhibitor (180). Cieri et al. showed an expansion of $CD25^+CD127^-FoxP3^+$ Tregs early after transplant, relative to the donor leukapheresis content and to the quantity in healthy subjects. Interestingly, patients who did not experience acute GVHD had a higher percentage of circulating Tregs at day +15 compared to patients who developed acute GVHD (180). Notably, the ability of Sirolimus to boost Treg reconstitution has also been reported outside of the PTCy platform. Indeed, Peccatori and colleagues reported an expansion of Treg after haplo-HCT using a combination of ATG, sirolimus and MMF as GVHD prophylaxis (190). Moreover, in the Baltimore experience with a cohort of patients undergoing MAC haplo-HCT using PTCy, MMF, and tacrolimus-based GVHD prophylaxis, Tregs achieved normal donor levels at all time-points examined (day +30, +90, +180, and +365) (181). Finally, in haplo-HCT using the GIAC protocol, patients with a higher day +30 percentage of naive Treg, defined as $CD4^+CD25^+CD45RA^+$, had a significantly lower incidence of grades II–IV acute GVHD (191). This highlights the importance of reaching a satisfactory Treg reconstitution for the achievement of immune tolerance after haplo-HCT.

γδ T Cells

γδ T cells combine conventional adaptive immunity features with innate-like MHC-independent tumor recognition (192). In healthy donors the majority of circulating γδ T cells expresses the Vδ2 chain, whereas the minority expresses the Vδ1 chain. The former subgroup is able to recognize non-peptide phosphoantigens and to perform direct killing of tumor cells (193). The Vδ1 γδ T-cell subgroup on the other hand is associated with control of CMV infection and also retains antitumor activity (194). Both subgroups play a key role in the setting of haplo-HCT because they do not induce GVHD but can exert immunological surveillance. In patients undergoing αβ-TCD haplo-HCT, γδ-T cells were the predominant T-cell subset for the first 2–3 weeks after transplant (91.5% of $CD3^+$ lymphocytes), while αβ T cells became the most prevalent population at 1 month (93). Moreover, patients had a higher proportion of γδ-T cells, especially the $V\delta2^+$ subset for the first 3 months. However, CMV reactivation (but not infection with other viruses) was associated with an expansion of Vδ1 γδ-T cells (93). Interestingly, the authors showed that zolendronic acid was able to potentiate $V\delta2^+$ killing against leukemia blasts after *in vitro* culture, indicating that the cytotoxicity was dependent on phosphoantigen recognition and providing a rationale for the development of future clinical trials to boost the γδ T anti-tumor effect (93). The same group tested the *in vivo* ability of zolendronic acid (ZOL) to enhance γδ T-cell recovery and function, by administrating the drug to pediatric patients undergoing αβ-TCR/CD19 depleted haplo-HCT. An induction of Vδ2-cell differentiation paralleled by increased cytotoxicity of both Vδ1 and Vδ2 cells against primary leukemia blasts was associated with ZOL treatment. Patients given three or more ZOL infusions had a better probability of survival in comparison to those given one or two treatments (86% vs. 54%, respectively, $p = 0.008$), suggesting that ZOL infusion promotes γδ T-cell differentiation and cytotoxicity and may influence the outcome of patients in this transplant setting (94).

B Cells

B cell recovery occurs late after HCT. B cells are almost undetectable during the first and second months and normal values are only reached around 12 months after transplant (195). In the setting of NMA haplo-BMT using PTCy, MMF and Tacrolimus as GVHD prophylaxis, B cells were undetectable until day +28. Recovery of B cells started from week 5 with an immature $CD38^{bright}$ $CD10^+$ Ki-67 negative phenotype, suggesting that the increase in B-cell number was not due to the homeostatic proliferation of transferred B cells but to *de novo* generation (196). Maturation of B cells was characterized by different expression of both transitional (T) markers CD5 and CD21: T0 ($CD5^-CD21^-$), T1 ($CD5^+CD21^-$), T2 ($CD5^+CD21^+$), and the $CD5^-CD21^+$ subset. Starting at week 9, mature B cells ($CD38^{dim}$ $CD10^-$) began to increase with a naïve phenotype (IgD^+, IgM^+). Overall, B cell maturation took 6 months to complete in the setting of a T-cell replete PTCy-based haplo-HCT (196).

With haplo-HCT using the GIAC protocol, median B cell counts did not differ from HLA-matched HCT at any of the time points examined (158). In an analysis comparing CD34 positive selection and CD3/CD19 cell depletion, B cells reconstituted more rapidly in the former group (156). Furthermore, recovery of B cells after αβ T cell-depleted haplo-HCT was delayed for the first 6 months compared to a cohort of patients transplanted with a MUD or MMUD using standard calcineurin-based GVHD prophylaxis. However, this is at least in part attributable to the fact that in the αβ T-cell depletion setting, patients received one dose of Rituximab as part of the conditioning regimen in order to prevent post-transplant lymphoproliferative disorders (97).

RELAPSE AND IMMUNE EVASION MECHANISMS AFTER HAPLO-HCT

Recent data has highlighted the critical role of the immune system in the control of myeloid leukemia after HCT and elucidated our understanding regarding the immunologic mechanisms underlying relapse after haplo-HCT. Work by Vago and colleagues revealed that a substantial proportion of AML and MDS relapses after haplo-HCT are attributable to acquired uniparental disomy of chromosome 6p (copy-neutral loss of heterozygosity eliminating the incompatible HLA alleles without decreasing the overall level of expression of HLA class I molecules). This was shown to result in loss of the mismatched HLA molecules on leukemia cells and immune escape from leukemia control exerted by haploidentical donor T cells via the major histocompatibility mismatch (197). The maintained overall expression of class I molecules in this study also evaded activation of NK-cell mediated anti-leukemic responses which could potentially be based on a newly missing ligand to an inhibitory KIR receptor (197). Clinical suspicion for an immune evasion phenomenon was first raised when patients relapsing after haplo-HCT had discrepant findings in host chimerism monitoring between short-tandem-repeat amplification but not HLA typing (198). Recognition of this leukemia escape mechanism has therapeutic importance for patients who are candidates for subsequent haplo-HCT in whom a different donor is available who is mismatched for the HLA haplotype retained in the relapsed leukemic cells and/or is predicted to mediate NK-cell alloreactivity based on the newly missing KIR-ligand. The development of routine diagnostic methods is expected to facilitate this (198). Importantly, ∼30% of relapses after haplo-HCT are attributable to this mechanism of the elimination of the incompatible HLA alleles irrespective of the GVHD prophylaxis or platform used to control T-cell alloreactivity (190, 199, 200).

To identify other drivers of post-HCT relapse Toffalori et al. analyzed transcriptional signatures specific for post-transplant AML relapses (201). This study demonstrated deregulation of the costimulatory interface between donor T cells and host leukemia cells, with loss of costimulatory interactions and enforcement of inhibitory ones (PD-1/PDL-1) as evidenced by both changes in leukemic cells and donor T cells (**Figure 8**). Additionally, the study documented downregulation of surface expression of HLA class II molecules on leukemia cells due to the downregulation of the HLA class II regulator CIITA (201). Patients with AML

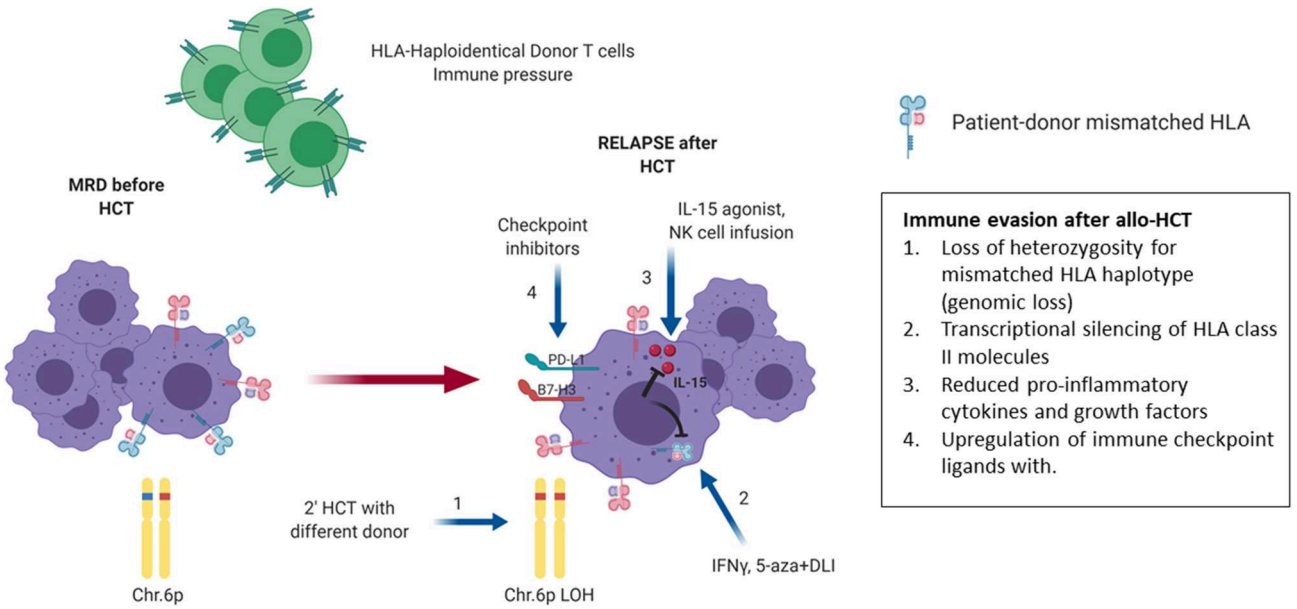

FIGURE 8 | Mechanisms of relapse post haploidentical HCT. Late relapse after haploidentical allogeneic transplantation can be driven by a number of immunologic mechanisms as shown. Under the immune pressure of graft-vs.-leukemia (GVL) via HLA mismatch in a haploidentical environment, loss of heterozygosity for the mismatched HLA allele is a mechanism of escape from immune surveillance and relapse (1). Another mechanism involves transcriptional silencing of HLA class II molecule, thereby reducing T-cell mediated GVL. This effect can be partially reversed in the presence of immunomodulatory molecules such as IFN-γ or the epigenetic regulator 5-azacitidine (5-aza) (2). Modification of the tumor microenvironment via suppression of release of mediators that promote GVL is another mechanism used by relapsing leukemia cells, which may be partially reversed via administration of IL-15 agonists and NK cell infusions that promote the secretion of proinflammatory cytokines (3). An additional common mechanism of relapse involves the emergence of T-cell exhaustion with associated upregulation of PD-L1 and other inhibitory receptors. The latter may be reversed through administration of checkpoint inhibitors (4). Blue arrows indicate possible therapeutic strategies to overcome the different mechanisms of immune evasion. MRD, minimal residual disease; LOH, loss of heterozygosity; Chr, chromosome; DLI, donor lymphocyte infusion. This Figure was created using BioRender.com.

relapse after HCT were found to have a higher proportion of BM—infiltrating T cells expressing inhibitory receptors (IR) compared to patients remaining in CR. The exhausted BM-T cell phenotype was associated with a restricted TCR repertoire, impaired effector functions and leukemia-reactive specificities. Furthermore, early detection of severely exhausted BM-memory stem T cells predicted relapse (202). Interestingly, IR-positive T cells infiltrating the BM of AML patients at relapse displayed a greater ability to recognize matched leukemic blasts after *in vitro* expansion compared with their IR-negative counterparts. This suggest that IR expression marks lymphocytes enriched for tumor specificity whose activity could be unleashed with therapeutic check-point blockade, although innovative targeted strategies will be required to avoid exacerbation of GVHD in the HCT context (202).

HAPLO-HCT AS A PLATFORM FOR POST-TRANSPLANT IMMUNE THERAPIES

Numerous scientific advances have contributed to the resurgence of haplo-HCT as a viable transplant option for patients requiring HCT and have achieved similar outcomes to those from other donor sources. The ability to perform haplo-HCT without costly *ex-vivo* T-cell depletion approaches, which require extensive cell manufacturing expertise frequently limited to large transplant centers, has been a major advance in transplant accessibility for patients in resource-limited countries that frequently do not perform unrelated donor transplantation (203). However, further efforts are required to improve immune reconstitution, control infectious complications and decrease relapse rates in patients after haplo-HCT. Fortunately, haplo-HCT provides an ideal platform characterized by unique immunologic properties and ready accessibility of the donor for additional cell products. This offers tremendous opportunities for the development and implementation of innovative adoptive immune cell therapies to augment infectious and antitumor immunity and further improve outcomes (**Figure 9**).

Suicide Mechanisms for Defined T-Cell Content in the Graft and Post-transplant

Ex-vivo TCD haplo-HCT affords opportunities not only for the dose-titration but also the manipulation of the T cell product prior to infusion into the patient. Rather than *in-* or *ex-vivo* approaches to selectively deplete or attenuate T cells, a different approach is the infusion of polyclonal T cells that have been genetically engineered to include an inducible suicide gene. With this strategy, a defined dose of T cells can be administered to aid in engraftment and immune reconstitution, mediate a GVL effect, and provide

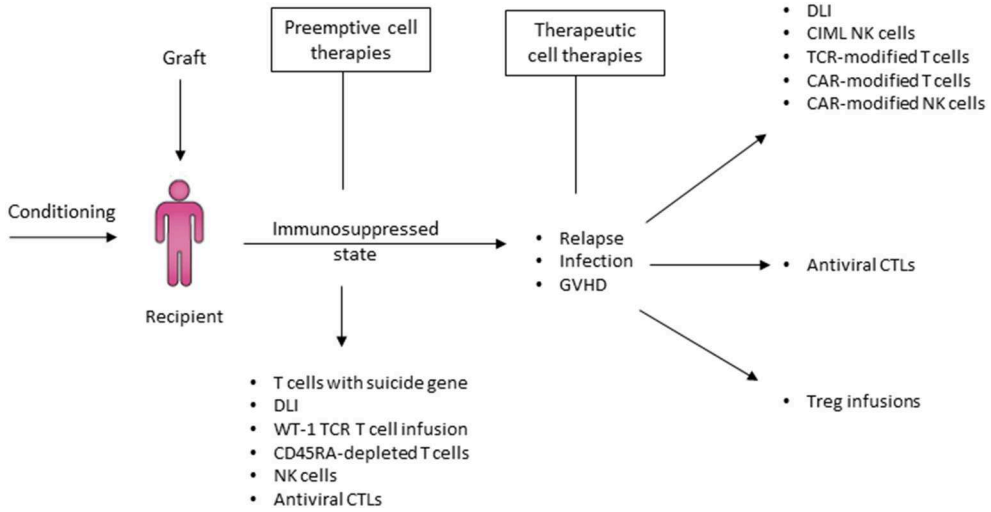

FIGURE 9 | Haplo-HCT offers a platform for post-transplant immune therapies to prevent and treat relapse. In the context of a haploidentical transplant, there are several options to administer cellular therapies in order to address relapse, infection and GVHD either pre-emptively or therapeutically. In the event of a relapse, enhancing GVL effect using cellular therapy that either relies on the haploidentical mismatch between donor and recipient or gene-modified donor immune effector cells T cells are potential options. Donors haploidentical to the recipient may also readily serve as a source of cells for the production of CAR-T or CAR-NK. In the event of significant viral infection post relapse, administration of antiviral cytotoxic T-cells may promote viral clearance without increasing the risk of GVHD. Finally, Treg infusions may be utilized to treat GVHD. DLI, Donor lymphocyte infusion; CIML NK cells, Cytokine-induced memory-like NK cells; TCR, T-cell receptor; CAR, Chimeric antigen receptor; CTLs, Cytotoxic T-lymphocytes.

infectious immunity while being selectively susceptible to an externally inducible suicide mechanism in the event of significant GVHD (204).

The first such approach was pioneered by Bonini and colleagues with the introduction of a herpes simplex virus thymidine kinase (HSV-TK) suicide gene into T cells using γ-retroviral transduction in which the transgene also contained the truncated selection marker ΔLNGFR. This allowed for the isolation and infusion of transduced cells bearing the suicide gene (205). With this strategy, administration of the drug ganciclovir activated the suicide mechanism and successfully controlled GVHD in several patients after infusion (204). Interestingly, the first wave of circulating TK^+ cells after infusion facilitated thymic renewal and was followed by a second wave of long-term immune reconstitution with naïve lymphocytes. This was supported by an increase in TCR excision circles, $CD31^+$ recent thymic emigrants and expansion of thymic tissue on imaging and was further associated with an increase in serum IL-7 levels following each infusion (206).

Since then, other approaches have been developed, such as transduction of T-cells with the iCasp-9 suicide gene. This gene can be activated by an otherwise inert drug (207, 208). Novel approaches have also included the use of a different transduction marker such as truncated CD19 that allows for the confirmation of transduction and if desired positive isolation of transduced T cells prior to infusion. Brenner and colleagues first utilized this approach in children undergoing haplo-HCT and demonstrated impressively how iCasp-9 transduced T cells expressing the truncated CD19 aid in immune reconstitution and contribute to infectious immunity (207, 208). Activation of the suicide gene led to resolution of GVHD symptoms within hours (209, 210). Interestingly, while alloreactivity was rapidly abrogated, suicide-gene transduced T cells were not permanently eliminated and able to reconstitute again without causing GVHD. Pediatric studies are underway to investigate suicide-gene equipped T-cell infusions after αβ-TCR/CD19 depleted haplo-HCT.

Haploidentical Donor Lymphocyte Infusions

A common approach to address relapse early after HCT is the infusion of donor lymphocyte infusions (DLI) to exert a GVL effect, but this is frequently accompanied by significant rates of GVHD. Zeidan and colleagues demonstrated the feasibility of this approach after haplo-HCT with PTCy in a retrospective analysis of a dose escalation approach at their center (211). Forty patients received 52 haplo-DLI doses initially at 1×10^5 $CD3^+$/kg and most commonly starting at 1×10^6 $CD3^+$/kg. Ten patients (25%) developed GVHD with Grade III-IV acute GVHD in 6 and chronic GVHD in 3 patients. Twelve patients (30%) achieved a CR with a median duration of 11.8 months (211).

Sun et al. reported on haplo-DLI following a number of different chemotherapy regimens (FLAG, Methotrexate and others) for relapse after haplo-HCT with the GIAC protocol. Of 86 patients, 20 developed Grade III-IV aGVHD and 41 developed cGHVD. NRM was 10.3%, and 62% of patients achieved a CR after chemo-DLI of which 50% experienced re-relapse at a median duration of 92 days (212). A modified GIAC backbone was also utilized to assess preemptive DLI at a median of 77 days post haplo-HCT in high risk patients to prevent relapse.

With a sizeable median $CD3^+$ dose of 1.8×10^7/kg, the 100-day incidences of acute GVHD were 55.3% for Grade II–IV and 10.2% for Grade III–IV, respectively. Two-year incidence of chronic GVHD was 52%, among which 18.2% were severe. With this regimen, 2-year NRM was high at 33.1% with a 2-year relapse incidence of 32% (213). Approaches to reduce GVHD while optimizing the GVL effect of preemptive or therapeutic DLI are likely to evolve over time and include the infusion of IL-10 anergized DLI (214), CD45-RA depleted DLI (215) and adoptive transfer of gene modified cells as described in this section. Although experience in the haplo-HCT setting is limited to date, azacitidine or decitabine in conjunction with DLI have shown promising overall response rates on the order of 25–33% for patients with AML or MDS relapsing after allogeneic HCT (216, 217).

CAR- T or CAR-NK-Cell Infusion

Chimeric antigen receptor (CAR) T cells targeting CD19 have revolutionized the treatment of relapsed/refractory B-cell acute lymphoblastic leukemias and aggressive B-cell lymphomas, with complete remission rates ranging from 70–90% in ALL (218, 219) and ∼60% for refractory large B-cell lymphoma (220, 221). CAR-T cells have been successfully manufactured from donor T cells in patients with relapse after allogeneic HCT and infused without mediating GVHD. Autoimmune complications have not been observed after infusion of CAR-T cells derived from autologous T cells suggesting that the CAR-signal overrides TCR-based recognition. The use of third-party CAR-T cells has been explored with concurrent transcription activator-like effector nuclease (TALEN)-based gene editing of the endogenous TCR. These CAR-T cells did mediate GVHD in a limited study of three patients (222). The use of CAR-NK cells is also being explored in the relapse setting, and although long-term persistence may be more limited than that of CAR-T cells, this approach may be beneficial when there is a higher degree of HLA-mismatch such as after haplo-HCT (223). While the therapeutic success of CD19-targeting CAR-T cell therapy to date is limited to B-cell malignancies and multiple myeloma (224), studies are underway to investigate the safety, feasibility, and preliminary efficacy of CAR-T cells directed against AML and MDS (225–227). Given this rapidly evolving field, the established efficacy potential of CAR-T cells and ability to utilize donor cells for CAR-T cell manufacture post-HCT, haploidentical HCT donors represent a readily available post-transplant cell source for donor-derived CAR-T cell or CAR-NK cell therapies for relapsed leukemia.

Antiviral Cytotoxic T Lymphocyte (CTL) Infusion

Infectious complications and particularly end-organ viral disease after HCT remain a challenge, particularly in haplo-HCT where *ex-* or *in-* vivo T-cell depletion is necessary. For example, the incidence of BK-virus hemorrhagic cystitis is higher in haplo-HCT (228). As demonstrated by Leen and Bollard the infusion of virus-specific CTL lines, generated by stimulating PBMC from adenovirus and EBV-seropositive donors, can safely be performed without inducing GVHD and can result in clearance of adenoviral disease and prevention of EBV-associated PTLD (229). The successful use of off-the-shelf multi-virus-specific T cells to treat viral infections after allogeneic HCT with minimal risk of GVHD has since been confirmed in a larger study and has the potential to mitigate serious viral disease after haplo-HCT either with third-party or haploidentical antiviral CTLs (230).

TCR-Edited T Cell Infusions

Whereas, CAR-transduced T cells recognize extracellular peptides on the surface of target cells in an MHC-independent manner, TCR-mediated T cell recognition mediates T cell immunity against MHC-restricted, intracellular targets and minor histocompatibility antigens. With the advent of sophisticated strategies to optimize T cell transduction and prevent mis-coupling of transduced and endogenous TCR chains, TCR-edited T cells have successfully entered clinical trials for patients with an HLA-type required for the HLA-restricted expression of the antigen. Greenberg and colleagues cloned a high affinity TCR targeting the HLA-A2 restricted tumor antigen WT-1 from healthy donors and inserted this TCR into EBV-specific donor $CD8^+$ T cells (to minimize the GVHD risk and enhance persistence). The WT1-TCR modified donor T cells were then infused prophylactically into the HLA-A*0201+ recipients after they had received an allogeneic HCT from the same donor. This approach resulted in 100% relapse free survival in the WT-1 TCR-T cell group at 44 months as compared to a comparative group of similar risk AML patients with a 54% relapse-free survival after HCT (231). A separate approach is currently under investigation to target the HLA-A*0201-restricted minor histocompatibility antigen HA-1, which is exclusively expressed on hematopoietic cells (232). When the immunogenic single-nucleotide polymorphic variant of HA-1 is expressed on hematopoietic cells of the HLA-A2+HCT-recipient, donor T cells that have been transduced to encode a high-avidity TCR recognizing HA-1 can effectively eliminate leukemia and lymphoma cells *in vitro* (233). Given the facile availability of donor T cells, haplo-HCT can and should serve as a beneficial platform to explore new approaches to reduce relapse after HCT.

NK Cell Product Infusion to Augment Graft vs. Tumor Effect

As previously described, NK-cells can mediate GVL effects due to KIR-mediated alloreactivity in the haplo-HCT setting. In addition to selecting the donor based on predicted NK-cell alloreactivity, the availability of haploidentical donors for additional cell product collection affords the unique opportunity to utilize NK-cell infusions to provide for additional GVL or GVT effects after HCT prophylactically or in the face of relapse (234). Generation of adequate numbers of NK cells for post-transplant therapies can be challenging given the relatively low NK cell frequency in the blood but can be overcome by *in vitro* expansion such as with membrane-bound IL-21 expressing feeder cells (mbIL21). A Phase 1 study evaluated prophylactic NK cell infusions after haplo-HCT with PTCy on days −2, +7, and +28. Of 11 enrolled patients who received all 3 planned NK cell doses, 54% developed Grade I-II aGVHD, and none developed Grade III-IV aGVHD, chronic GVHD or dose-limiting toxicities.

Only 1/11 patient relapsed. All others were alive and in remission at a median follow-up of 14.7 months (235). Administration of cytokines can facilitate NK cell expansion, but certain cytokines such as IL-2 also preferentially expand Tregs based on their constitutive expression of high-affinity IL-2R (CD25). These Tregs in turn inhibit NK cell proliferation (236). A study treating AML patients with haploidentical NK cell infusions after lymphodepletion with cyclophosphamide and fludarabine demonstrated that NK cell expansion was most pronounced and effective when IL-2-diphteria toxin fusion protein was administered to achieve host Treg depletion (237).

A recent trial administering haploidentical NK cells with rhIL15 for relapsed AML after lymphodepleting chemotherapy showed that rhIL-15 achieved better rates of *in vivo* NK-cell expansion and remission compared to previous trials utilizing IL-2, but also observed steroid- and tocilizumab-responsive cytokine release syndrome and neurologic toxicity which was associated with high levels of IL-6 (238). Cytokine-induced memory-like (CIML) NK cells from haploidentical donors were able to induce complete remissions in relapsed/refractory AML patients outside of the transplant setting without any toxicities (239). This GVL effect may be even more durable when NK cells from the same haploidentical donor are infused after haplo-HCT because no immunologic rejection of the CIML NK cells from the same donor is expected. Studies to date have suggested that KIR-reactivity is less important when NK cells are cytokine-induced (240). Studies are now underway to evaluate the safety and efficacy of CIML NK cells for relapse after haplo-HCT.

Cytokine Support to Enhance NK-Cell Alloreactivity After Hct

An alternative strategy to address relapse after HCT is the administration of cytokines aimed at enhancing the anti-leukemic function of the existing post-transplant immune environment. One such approach employed ALT-803, an IL-15 superagonist complex designed to extend the *in vivo* half-life of IL-15 and mimic the physiologic *trans*-presentation of IL-15 (241). In contrast to IL-2 that can promote the survival, proliferation, and activation of lymphocytes, but that also stimulates Tregs, IL-15 preferentially expands $CD8^+$ T cells and NK cells via trans-presentation to the IL-2/15R$\beta\gamma_c$-receptor while avoiding the stimulation of Tregs. In a recent Phase 1 trial ALT-803 was well-tolerated, particularly when administered subcutaneously, and induced responses of 19% in patients relapsed after HCT (241), suggesting that such agents may also be explored in the haplo-HCT setting. Efforts are underway to test use of IL-15 or IL-15 superagonist complex alone or in combination with NK cell- based therapy to target relapse after haplo-HCT.

CONCLUSION

The initial immunologic barriers to haplo-HCT, namely GVHD and graft failure, have been overcome with different platforms that can be utilized to control T cell alloreactivity post-transplant. Comparable clinical outcomes have now been achieved relative to alternative donor sources and depending on the specific scenario, haplo-HCT can offer a lower risk of GVHD and/or improved control against relapse. The GVL effect in haplo-HCT is particularly intriguing given the concept of NK-cell alloreactivity based on the KIR/KIR-ligand system and ability to select donors accordingly. An emerging body of literature is elucidating immunologic mechanisms of GVHD and relapse that are potentially targetable and highlight the immune pressure exerted by donor immune cells after HCT. Given ready accessibility of the donor, haplo-HCT offers a unique platform for post-transplant cell-based immune therapies aimed at expediting immune reconstitution, improving thymic function, providing infectious immunity, and treating or protecting against relapse, while maintaining therapeutic control of those cell immunotherapies with methods such as suicide mechanisms. The rapid advancements in our understanding of the immunobiology of haplo-HCT are therefore poised to lead to increasingly sophisticated strategies to fine-tune the transplant process and to further improve outcomes after haplo-HCT.

AUTHOR CONTRIBUTIONS

SB, BR, RS, and RR helped review the literature and wrote this manuscript.

REFERENCES

1. Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med.* (2006) 354:1813–26. doi: 10.1056/NEJMra052638
2. Gragert L, Eapen M, Williams E, Freeman J, Spellman S, Baitty R, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med.* (2014) 371:339–48. doi: 10.1056/NEJMsa1311707
3. Morishima Y, Kashiwase K, Matsuo K, Azuma F, Morishima S, Onizuka M, et al. Biological significance of HLA locus matching in unrelated donor bone marrow transplantation. *Blood.* (2015) 125:1189–97. doi: 10.1182/blood-2014-10-604785
4. Fuchs EJ. Haploidentical transplantation for hematologic malignancies: where do we stand? *Hematology Am Soc Hematol Educ Program.* (2012) 2012:230–6. doi: 10.1182/asheducation.V2012.1.230.3798312
5. Powles RL, Morgenstern GR, Kay HE, McElwain TJ, Clink HM, Dady PJ, et al. Mismatched family donors for bone-marrow transplantation as treatment for acute leukaemia. *Lancet.* (1983) 1:612–5. doi: 10.1016/S0140-6736(83)91793-2
6. Beatty PG, Clift RA, Mickelson EM, Nisperos BB, Flournoy N, Martin PJ, et al. Marrow transplantation from related donors other than HLA-identical siblings. *N Engl J Med.* (1985) 313:765–71. doi: 10.1056/NEJM198509263131301
7. Anasetti C, Beatty PG, Storb R, Martin PJ, Mori M, Sanders JE, et al. Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. *Hum Immunol.* (1990) 29:79–91. doi: 10.1016/0198-8859(90)90071-V
8. Reisner Y, Kapoor N, Kirkpatrick D, Pollack MS, Cunningham-Rundles S, Dupont B, et al. Transplantation for severe combined immunodeficiency with HLA-A,B,D,DR incompatible parental marrow cells fractionated by

soybean agglutinin and sheep red blood cells. *Blood.* (1983) 61:341–8. doi: 10.1182/blood.V61.2.341.341
9. Bachar-Lustig E, Rachamim N, Li HW, Lan F, Reisner Y. Megadose of T cell-depleted bone marrow overcomes MHC barriers in sublethally irradiated mice. *Nat Med.* (1995) 1:1268–73. doi: 10.1038/nm1295-1268
10. Reisner Y, Bachar-Lustig E, Li HW, Aversa F, Velardi A, Martelli MF. The role of megadose CD34+ progenitor cells in the treatment of leukemia patients without a matched donor and in tolerance induction for organ transplantation. *Ann N Y Acad Sci.* (1999) 872:336–48; discussion 48–50. doi: 10.1111/j.1749-6632.1999.tb08478.x
11. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
12. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W, et al. Haploidentical hematopoietic stem cell transplantation without *in vitro* T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant.* (2006) 38:291–7. doi: 10.1038/sj.bmt.1705445
13. Mavers M, Bertaina A. High-risk leukemia: past, present, and future role of NK cells. *J Immunol Res.* (2018) 2018:1586905. doi: 10.1155/2018/1586905
14. Gill S, Olson JA, Negrin RS. Natural killer cells in allogeneic transplantation: effect on engraftment, graft- versus-tumor, and graft-versus-host responses. *Biol Blood Marrow Transplant.* (2009) 15:765–76. doi: 10.1016/j.bbmt.2009.01.019
15. Lupo KB, Matosevic S. Natural killer cells as allogeneic effectors in adoptive cancer immunotherapy. *Cancers.* (2019) 11:769. doi: 10.3390/cancers11060769
16. Lee CJ, Savani BN, Mohty M, Labopin M, Ruggeri A, Schmid C, et al. Haploidentical hematopoietic cell transplantation for adult acute myeloid leukemia: a position statement from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Haematologica.* (2017) 102:1810–22. doi: 10.3324/haematol.2017.176107
17. Zhang P, Tey SK. Adoptive T cell therapy following haploidentical hematopoietic stem cell transplantation. *Front Immunol.* (2019) 10:1854. doi: 10.3389/fimmu.2019.01854
18. Ottaviano G, Chiesa R, Feuchtinger T, Vickers MA, Dickinson A, Gennery AR, et al. Adoptive T cell therapy strategies for viral infections in patients receiving haematopoietic stem cell transplantation. *Cells.* (2019) 8:47. doi: 10.3390/cells8010047
19. Henslee PJ, Thompson JS, Romond EH, Doukas MA, Metcalfe M, Marshall ME, et al. T cell depletion of HLA and haploidentical marrow reduces graft-versus-host disease but it may impair a graft-versus-leukemia effect. *Transplant Proc.* (1987) 19(1 Pt 3):2701–6.
20. Szydlo R, Goldman JM, Klein JP, Gale RP, Ash RC, Bach FH, et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *J Clin Oncol.* (1997) 15:1767–77. doi: 10.1200/JCO.1997.15.5.1767
21. Kernan NA, Collins NH, Juliano L, Cartagena T, Dupont B, O'Reilly RJ. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. *Blood.* (1986) 68:770–3. doi: 10.1182/blood.V68.3.770.770
22. Kernan NA, Flomenberg N, Dupont B, O'Reilly RJ. Graft rejection in recipients of T-cell-depleted HLA-nonidentical marrow transplants for leukemia. Identification of host-derived antidonor allocytotoxic T lymphocytes. *Transplantation.* (1987) 43:842–7. doi: 10.1097/00007890-198743060-00014
23. Reisner Y, Ben-Bassat I, Douer D, Kaploon A, Schwartz E, Ramot B. Demonstration of clonable alloreactive host T cells in a primate model for bone marrow transplantation. *Proc Natl Acad Sci USA.* (1986) 83:4012–5. doi: 10.1073/pnas.83.11.4012
24. Ash RC, Horowitz MM, Gale RP, van Bekkum DW, Casper JT, Gordon-Smith EC, et al. Bone marrow transplantation from related donors other than HLA-identical siblings: effect of T cell depletion. *Bone Marrow Transplant.* (1991) 7:443–52.
25. O'Reilly RJ, Keever C, Kernan NA, Brochstein J, Collins N, Flomenberg N, et al. HLA nonidentical T cell depleted marrow transplants: a comparison of results in patients treated for leukemia and severe combined immunodeficiency disease. *Transplant Proc.* (1987) 19 (6 Suppl 7):55–60.
26. Anasetti C, Amos D, Beatty PG, Appelbaum FR, Bensinger W, Buckner CD, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med.* (1989) 320:197–204. doi: 10.1056/NEJM198901263200401
27. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol.* (2005) 23:3447–54. doi: 10.1200/JCO.2005.09.117
28. Aversa F, Tabilio A, Velardi A, Cunningham I, Terenzi A, Falzetti F, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med.* (1998) 339:1186–93. doi: 10.1056/NEJM199810223391702
29. Rachamim N, Gan J, Segall H, Krauthgamer R, Marcus H, Berrebi A, et al. Tolerance induction by "megadose" hematopoietic transplants: donor-type human CD34 stem cells induce potent specific reduction of host anti-donor cytotoxic T lymphocyte precursors in mixed lymphocyte culture. *Transplantation.* (1998) 65:1386–93. doi: 10.1097/00007890-199805270-00017
30. Gur H, Krauthgamer R, Bachar-Lustig E, Katchman H, Arbel-Goren R, Berrebi A, et al. Immune regulatory activity of CD34+ progenitor cells: evidence for a deletion-based mechanism mediated by TNF-alpha. *Blood.* (2005) 105:2585–93. doi: 10.1182/blood-2002-11-3463
31. Terenzi A, Aristei C, Aversa F, Perruccio K, Chionne F, Raymondi C, et al. Efficacy of fludarabine as an immunosuppressor for bone marrow transplantation conditioning: preliminary results. *Transplant Proc.* (1996) 28:3101.
32. Aversa F, Tabilio A, Terenzi A, Velardi A, Falzetti F, Giannoni C, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. *Blood.* (1994) 84:3948–55. doi: 10.1182/blood.V84.11.3948.bloodjournal84113948
33. Berenbaum MC, Brown IN. Prolongation of homograft survival in mice with single doses of cyclophosphamide. *Nature.* (1963) 200:84. doi: 10.1038/200084a0
34. Colson YL, Li H, Boggs SS, Patrene KD, Johnson PC, Ildstad ST. Durable mixed allogeneic chimerism and tolerance by a nonlethal radiation-based cytoreductive approach. *J Immunol.* (1996) 157:2820–9.
35. Colson YL, Wren SM, Schuchert MJ, Patrene KD, Johnson PC, Boggs SS, et al. A nonlethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *J Immunol.* (1995) 155:4179–88.
36. Robinson TM, O'Donnell PV, Fuchs EJ, Luznik L. Haploidentical bone marrow and stem cell transplantation: experience with post-transplantation cyclophosphamide. *Semin Hematol.* (2016) 53:90–7. doi: 10.1053/j.seminhematol.2016.01.005
37. Nomoto K, Eto M, Yanaga K, Nishimura Y, Maeda T, Nomoto K. Interference with cyclophosphamide-induced skin allograft tolerance by cyclosporin A. *J Immunol.* (1992) 149:2668–74.
38. Dukor P, Dietrich FM. Prevention of cyclophosphamide-induced tolerance to erythrocytes by pretreatment with cortisone. *Proc Soc Exp Biol Med.* (1970) 133:280–5. doi: 10.3181/00379727-133-34456
39. Ross D, Jones M, Komanduri K, Levy RB. Antigen and lymphopenia-driven donor T cells are differentially diminished by post-transplantation administration of cyclophosphamide after hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* (2013) 19:1430–8. doi: 10.1016/j.bbmt.2013.06.019
40. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Maeda T, et al. Specific destruction of host-reactive mature T cells of donor origin prevents graft-versus-host disease in cyclophosphamide-induced tolerant mice. *J Immunol.* (1991) 146:1402–9.
41. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Nomoto K. The requirement of intrathymic mixed chimerism and clonal deletion for a long-lasting skin allograft tolerance in cyclophosphamide-induced tolerance. *Eur J Immunol.* (1990) 20:2005–13. doi: 10.1002/eji.1830200919

42. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Nomoto K. Sequential mechanisms of cyclophosphamide-induced skin allograft tolerance including the intrathymic clonal deletion followed by late breakdown of the clonal deletion. *J Immunol.* (1990) 145:1303–10.
43. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nomoto K. Intrathymic clonal deletion of V beta 6+ T cells in cyclophosphamide-induced tolerance to H-2-compatible, Mls-disparate antigens. *J Exp Med.* (1990) 171:97–113. doi: 10.1084/jem.171.1.97
44. McCurdy SR, Luznik L. Post-transplantation cyclophosphamide for chimerism-based tolerance. *Bone Marrow Transplant.* (2019) 54 (Suppl 2):769–74. doi: 10.1038/s41409-019-0615-0
45. Kong YY, Eto M, Omoto K, Umesue M, Hashimoto A, Nomoto K. Regulatory T cells in maintenance and reversal of peripheral tolerance *in vivo*. *J Immunol.* (1996) 157:5284–9.
46. Tomita Y, Mayumi H, Eto M, Nomoto K. Importance of suppressor T cells in cyclophosphamide-induced tolerance to the non-H-2-encoded alloantigens. Is mixed chimerism really required in maintaining a skin allograft tolerance? *J Immunol.* (1990) 144:463–73.
47. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. *J Clin Invest.* (2019) 130:2357–73. doi: 10.1172/JCI124218
48. Kanakry CG, Ganguly S, Zahurak M, Bolaños-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. *Sci Transl Med.* (2013) 5:211ra157. doi: 10.1126/scitranslmed.3006960
49. Ganguly S, Ross DB, Panoskaltsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, et al. Donor CD4+ Foxp3+ regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. *Blood.* (2014) 124:2131–41. doi: 10.1182/blood-2013-10-525873
50. O'Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, Phelps M, et al. Nonmyeloablative bone marrow transplantation from partially HLA-mismatched related donors using posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2002) 8:377–86. doi: 10.1053/bbmt.2002.v8.pm12171484
51. Chang YJ, Huang XJ. Haploidentical stem cell transplantation: anti-thymocyte globulin-based experience. *Semin Hematol.* (2016) 53:82–9. doi: 10.1053/j.seminhematol.2016.01.004
52. Korbling M, Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? *Blood.* (2001) 98:2900–8. doi: 10.1182/blood.V98.10.2900
53. Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med.* (2012) 367:1487–96. doi: 10.1056/NEJMoa1203517
54. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C. Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. *Blood.* (2000) 95:2484–90. doi: 10.1182/blood.V95.8.2484
55. Pan L, Delmonte J Jr, Jalonen CK, Ferrara JL. Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-versus-host disease. *Blood.* (1995) 86:4422–9. doi: 10.1182/blood.V86.12.4422.bloodjournal86124422
56. Mielcarek M, Martin PJ, Torok-Storb B. Suppression of alloantigen-induced T-cell proliferation by CD14+ cells derived from granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells. *Blood.* (1997) 89:1629–34. doi: 10.1182/blood.V89.5.1629
57. Tanaka J, Mielcarek M, Torok-Storb B. Impaired induction of the CD28-responsive complex in granulocyte colony-stimulating factor mobilized CD4 T cells. *Blood.* (1998) 91:347–52. doi: 10.1182/blood.V91.1.347
58. Mielcarek M, Graf L, Johnson G, Torok-Storb B. Production of interleukin-10 by granulocyte colony-stimulating factor-mobilized blood products: a mechanism for monocyte-mediated suppression of T-cell proliferation. *Blood.* (1998) 92:215–22. doi: 10.1182/blood.V92.1.215.413k10_215_222
59. Morton J, Hutchins C, Durrant S. Granulocyte-colony-stimulating factor (G-CSF)-primed allogeneic bone marrow: significantly less graft-versus-host disease and comparable engraftment to G-CSF-mobilized peripheral blood stem cells. *Blood.* (2001) 98:3186–91. doi: 10.1182/blood.V98.12.3186
60. Huang B, Hirst AR, Smith DK, Castelletto V, Hamley IW. A direct comparison of one- and two-component dendritic self-assembled materials: elucidating molecular recognition pathways. *J Am Chem Soc.* (2005) 127:7130–9. doi: 10.1021/ja050412d
61. Chen SH, Li X, Huang XJ. Effect of recombinant human granulocyte colony-stimulating factor on T-lymphocyte function and the mechanism of this effect. *Int J Hematol.* (2004) 79:178–84. doi: 10.1532/IJH97.A10227
62. Zhao XY, Chang YJ, Huang XJ. Effects of rhG-CSF mobilization on immunological properties of grafts from peripheral blood and bone marrow. *Zhongguo Shi Yan Xue Ye Xue Za Zhi.* (2006) 14:787–90.
63. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, et al. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. *Blood.* (2015) 125:3956–62. doi: 10.1182/blood-2015-02-627786
64. Arcese W, Picardi A, Santarone S, De Angelis G, Cerretti R, Cudillo L, et al. Haploidentical, G-CSF-primed, unmanipulated bone marrow transplantation for patients with high-risk hematological malignancies: an update. *Bone Marrow Transplant.* (2015)50 (Suppl 2):S24–30. doi: 10.1038/bmt.2015.91
65. Wang Y, Chang YJ, Xu LP, Liu KY, Liu DH, Zhang XH, et al. Who is the best donor for a related HLA haplotype-mismatched transplant? *Blood.* (2014) 124:843–50. doi: 10.1182/blood-2014-03-563130
66. Reisner Y, Kapoor N, O'Reilly RJ, Good RA. Allogeneic bone marrow transplantation using stem cells fractionated by lectins: VI, *in vitro* analysis of human and monkey bone marrow cells fractionated by sheep red blood cells and soybean agglutinin. *Lancet.* (1980) 2:1320–4. doi: 10.1016/S0140-6736(80)92394-6
67. Reisner Y, Itzicovitch L, Meshorer A, Sharon N. Hemopoietic stem cell transplantation using mouse bone marrow and spleen cells fractionated by lectins. *Proc Natl Acad Sci USA.* (1978) 75:2933–6. doi: 10.1073/pnas.75.6.2933
68. Reisner Y, Kapoor N, Kirkpatrick D, Pollack MS, Dupont B, Good RA, et al. Transplantation for acute leukaemia with HLA-A and B nonidentical parental marrow cells fractionated with soybean agglutinin and sheep red blood cells. *Lancet.* (1981) 2:327–31. doi: 10.1016/S0140-6736(81)90647-4
69. Civin CI, Strauss LC, Fackler MJ, Trischmann TM, Wiley JM, Loken MR. Positive stem cell selection–basic science. *Prog Clin Biol Res.* (1990) 333:387–401; discussion 2.
70. Heimfeld S, Fogarty B, McGuire K, Williams S, Berenson RJ. Peripheral blood stem cell mobilization after stem cell factor or G-CSF treatment: rapid enrichment for stem and progenitor cells using the CEPRATE immunoaffinity separation system. *Transplant Proc.* (1992) 24:2818.
71. Handgretinger R, Dopfer R, Hammer S, Lang P, Daurer B, Kimmig A, et al. The efficiency of a new cell separation technique in bone marrow purging without damaging the purged tumor cells. *Prog Clin Biol Res.* (1990) 333:303–9.
72. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science.* (2002) 295:2097–100. doi: 10.1126/science.1068440
73. Handgretinger R, Klingebiel T, Lang P, Schumm M, Neu S, Geiselhart A, et al. Megadose transplantation of purified peripheral blood CD34(+) progenitor cells from HLA-mismatched parental donors in children. *Bone Marrow Transplant.* (2001) 27:777–83. doi: 10.1038/sj.bmt.1702996
74. Feuchtinger T, Richard C, Pfeiffer M, Neuhauser F, Lucke J, Handgretinger R, et al. Adenoviral infections after transplantation of positive selected stem cells from haploidentical donors in children: an update. *Klin Padiatr.* (2005) 217:339–44. doi: 10.1055/s-2005-872530
75. Lang P, Schumm M, Greil J, Bader P, Klingebiel T, Muller I, et al. A comparison between three graft manipulation methods for haploidentical stem cell transplantation in pediatric patients: preliminary results of a pilot study. *Klin Padiatr.* (2005) 217:334–8. doi: 10.1055/s-2005-872529
76. McManigle W, Youssef A, Sarantopoulos S. B cells in chronic graft-versus-host disease. *Hum Immunol.* (2019) 80:393–9. doi: 10.1016/j.humimm.2019.03.003
77. Liu L, Zhang X, Feng S. Epstein-barr virus-related post-transplantation lymphoproliferative disorders after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2018) 24:1341–9. doi: 10.1016/j.bbmt.2018.02.026

78. Burns DM, Rana S, Martin E, Nagra S, Ward J, Osman H, et al. Greatly reduced risk of EBV reactivation in rituximab-experienced recipients of alemtuzumab-conditioned allogeneic HSCT. *Bone Marrow Transplant.* (2016) 51:825–32. doi: 10.1038/bmt.2016.19
79. Federmann B, Bornhauser M, Meisner C, Kordelas L, Beelen DW, Stuhler G, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: a phase II study. *Haematologica.* (2012) 97:1523–31. doi: 10.3324/haematol.2011.059378
80. Bethge WA, Faul C, Bornhäuser M, Stuhler G, Beelen DW, Lang P, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: an update. *Blood Cells Mol Dis.* (2008) 40:13–9. doi: 10.1016/j.bcmd.2007.07.001
81. Handgretinger R, Schilbach K. The potential role of gammadelta T cells after allogeneic HCT for leukemia. *Blood.* (2018) 131:1063–72. doi: 10.1182/blood-2017-08-752162
82. Drobyski WR, Majewski D, Hanson G. Graft-facilitating doses of *ex vivo* activated gammadelta T cells do not cause lethal murine graft-vs.-host disease. *Biol Blood Marrow Transplant.* (1999) 5:222–30. doi: 10.1053/bbmt.1999.v5.pm10465102
83. Bendelac A, Bonneville M, Kearney JF. Autoreactivity by design: innate B and T lymphocytes. *Nat Rev Immunol.* (2001) 1:177–86. doi: 10.1038/35105052
84. Hayday AC. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity.* (2009) 31:184–96. doi: 10.1016/j.immuni.2009.08.006
85. Viey E, Laplace C, Escudier B. Peripheral gammadelta T-lymphocytes as an innovative tool in immunotherapy for metastatic renal cell carcinoma. *Expert Rev Anticancer Ther.* (2005) 5:973–86. doi: 10.1586/14737140.5.6.973
86. Corvaisier M, Moreau-Aubry A, Diez E, Bennouna J, Mosnier JF, Scotet E, et al. V gamma 9V delta 2 T cell response to colon carcinoma cells. *J Immunol.* (2005) 175:5481–8. doi: 10.4049/jimmunol.175.8.5481
87. Bonneville M, Scotet E. Synergism and complementarity between human CD1 AND MHC-restricted T cells, two lymphoid subsets directed against distinct antigenic worlds. *Front Biosci.* (2005) 10:596–607. doi: 10.2741/1556
88. Dieli F, Troye-Blomberg M, Ivanyi J, Fournie JJ, Krensky AM, Bonneville M, et al. Granulysin-dependent killing of intracellular and extracellular *Mycobacterium tuberculosis* by Vgamma9/Vdelta2 T lymphocytes. *J Infect Dis.* (2001) 184:1082–5. doi: 10.1086/323600
89. Dudal S, Turriere C, Bessoles S, Fontes P, Sanchez F, Liautard J, et al. Release of LL-37 by activated human Vgamma9Vdelta2 T cells: a microbicidal weapon against Brucella suis. *J Immunol.* (2006) 177:5533–9. doi: 10.4049/jimmunol.177.8.5533
90. Perko R, Kang G, Sunkara A, Leung W, Thomas PG, Dallas MH. Gamma delta T cell reconstitution is associated with fewer infections and improved event-free survival after hematopoietic stem cell transplantation for pediatric leukemia. *Biol Blood Marrow Transplant.* (2015) 21:130–6. doi: 10.1016/j.bbmt.2014.09.027
91. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Abhyankar S, et al. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. *Bone Marrow Transplant.* (2007) 39:751–7. doi: 10.1038/sj.bmt.1705650
92. Lamb LS Jr, Henslee-Downey PJ, Parrish RS, Godder K, Thompson J, Lee C, et al. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. *J Hematother.* (1996) 5:503–9. doi: 10.1089/scd.1.1996.5.503
93. Airoldi I, Bertaina A, Prigione I, Zorzoli A, Pagliara D, Cocco C, et al. γδ T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-αβ+/CD19+ lymphocytes. *Blood.* (2015) 125:2349–58. doi: 10.1182/blood-2014-09-599423
94. Bertaina A, Zorzoli A, Petretto A, Barbarito G, Inglese E, Merli P, et al. Zoledronic acid boosts gammadelta T-cell activity in children receiving alphabeta(+) T and CD19(+) cell-depleted grafts from an HLA-haplo-identical donor. *Oncoimmunology.* (2017) 6:e1216291. doi: 10.1080/2162402X.2016.1216291
95. Locatelli F, Merli P, Pagliara D, Li Pira G, Falco M, Pende D, et al. Outcome of children with acute leukemia given HLA-haploidentical HSCT after alphabeta T-cell and B-cell depletion. *Blood.* (2017) 130:677–85. doi: 10.1182/blood-2017-04-779769
96. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, et al. HLA-haploidentical stem cell transplantation after removal of alphabeta+ T and B cells in children with nonmalignant disorders. *Blood.* (2014) 124:822–6. doi: 10.1182/blood-2014-03-563817
97. Bertaina A, Zecca M, Buldini B, Sacchi N, Algeri M, Saglio F, et al. Unrelated donor vs HLA-haploidentical alpha/beta T-cell- and B-cell-depleted HSCT in children with acute leukemia. *Blood.* (2018) 132:2594–607. doi: 10.1182/blood-2018-07-861575
98. Lang P, Handgretinger R, Meisel R, Mielke S, Niederwieser D, Schlegel PG, et al. Results of a prospective, multicenter, phase I/II clinical study in pediatric and adult patients using TCR alpha/beta and CD19 depleted haploidentical hematopoietic stem cell grafts following reduced-intensity conditioning. *Blood.* (2018) 132 (Suppl 1):604. doi: 10.1182/blood-2018-99-110124
99. Restifo NP, Gattinoni L. Lineage relationship of effector and memory T cells. *Curr Opin Immunol.* (2013) 25:556–63. doi: 10.1016/j.coi.2013.09.003
100. Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science.* (2000) 290:92–7. doi: 10.1126/science.290.5489.92
101. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, et al. Memory CD4+ T cells do not induce graft-versus-host disease. *J Clin Invest.* (2003) 112:101–8. doi: 10.1172/JCI17601
102. Zheng H, Matte-Martone C, Li H, Anderson BE, Venketesan S, Sheng Tan H, et al. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. *Blood.* (2008) 111:2476–84. doi: 10.1182/blood-2007-08-109678
103. Zheng H, Matte-Martone C, Jain D, McNiff J, Shlomchik WD. Central memory CD8+ T cells induce graft-versus-host disease and mediate graft-versus-leukemia. *J Immunol.* (2009) 182:5938–48. doi: 10.4049/jimmunol.0802212
104. Dutt S, Tseng D, Ermann J, George TI, Liu YP, Davis CR, et al. Naive and memory T cells induce different types of graft-versus-host disease. *J Immunol.* (2007) 179:6547–54. doi: 10.4049/jimmunol.179.10.6547
105. Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L- memory T cells without graft-versus-host disease. *Blood.* (2004) 103:1534–41. doi: 10.1182/blood-2003-08-2987
106. Li N, Matte-Martone C, Zheng H, Cui W, Venkatesan S, Tan HS, et al. Memory T cells from minor histocompatibility antigen-vaccinated and virus-immune donors improve GVL and immune reconstitution. *Blood.* (2011) 118:5965–76. doi: 10.1182/blood-2011-07-367011
107. Bleakley M, Heimfeld S, Jones LA, Turtle C, Krause D, Riddell SR, et al. Engineering human peripheral blood stem cell grafts that are depleted of naive T cells and retain functional pathogen-specific memory T cells. *Biol Blood Marrow Transplant.* (2014) 20:705–16. doi: 10.1016/j.bbmt.2014.01.032
108. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. *J Clin Invest.* (2015) 125:2677–89. doi: 10.1172/JCI81229
109. Triplett BM, Shook DR, Eldridge P, Li Y, Kang G, Dallas M, et al. Rapid memory T-cell reconstitution recapitulating CD45RA-depleted haploidentical transplant graft content in patients with hematologic malignancies. *Bone Marrow Transplant.* (2015) 50:1012. doi: 10.1038/bmt.2015.139
110. Touzot F, Neven B, Dal-Cortivo L, Gabrion A, Moshous D, Cros G, et al. CD45RA depletion in HLA-mismatched allogeneic hematopoietic stem cell transplantation for primary combined immunodeficiency: a preliminary study. *J Allergy Clin Immunol.* (2015) 135:1303–9.e1–3. doi: 10.1016/j.jaci.2014.08.019
111. Gribben JG, Guinan EC, Boussiotis VA, Ke XY, Linsley L, Sieff C, et al. Complete blockade of B7 family-mediated costimulation is necessary to induce human alloantigen-specific anergy: a method to ameliorate graft-versus-host disease and extend the donor pool. *Blood.* (1996) 87:4887–93. doi: 10.1182/blood.V87.11.4887.bloodjournal87114887
112. Guinan EC, Boussiotis VA, Neuberg D, Brennan LL, Hirano N, Nadler LM, et al. Transplantation of anergic histoincompatible bone marrow allografts. *N Engl J Med.* (1999) 340:1704–14. doi: 10.1056/NEJM199906033402202

113. Bastien JP, Krosl G, Therien C, Rashkovan M, Scotto C, Cohen S, et al. Photodepletion differentially affects CD4+ Tregs versus CD4+ effector T cells from patients with chronic graft-versus-host disease. *Blood.* (2010) 116:4859–69. doi: 10.1182/blood-2010-03-273193
114. Perruccio K, Topini F, Tosti A, Carotti A, Burchielli E, Ruggeri L, et al. Optimizing a photoallodepletion protocol for adoptive immunotherapy after haploidentical SCT. *Bone Marrow Transplant.* (2012) 47:1196–200. doi: 10.1038/bmt.2011.237
115. Perruccio K, Topini F, Tosti A, Carotti A, Aloisi T, Aversa F, et al. Photodynamic purging of alloreactive T cells for adoptive immunotherapy after haploidentical stem cell transplantation. *Blood Cells Mol Dis.* (2008) 40:76–83. doi: 10.1016/j.bcmd.2007.06.022
116. Roy DC, Lachance S, Cohen S, Delisle JS, Kiss T, Sauvageau G, et al. Allodepleted T-cell immunotherapy after haploidentical haematopoietic stem cell transplantation without severe acute graft-versus-host disease (GVHD) in the absence of GVHD prophylaxis. *Br J Haematol.* (2019) 186:754–66. doi: 10.1111/bjh.15970
117. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature.* (2009) 457:557–61. doi: 10.1038/nature07665
118. Vacca P, Montaldo E, Croxatto D, Moretta F, Bertaina A, Vitale C, et al. NK cells and other innate lymphoid cells in hematopoietic stem cell transplantation. *Front Immunol.* (2016) 7:188. doi: 10.3389/fimmu.2016.00188
119. Freud AG, Mundy-Bosse BL, Yu J, Caligiuri MA. The broad spectrum of human natural killer cell diversity. *Immunity.* (2017) 47:820–33. doi: 10.1016/j.immuni.2017.10.008
120. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science.* (2011) 331:44–9. doi: 10.1126/science.1198687
121. Lanier LL. NKG2D receptor and its ligands in host defense. *Cancer Immunol Res.* (2015) 3:575–82. doi: 10.1158/2326-6066.CIR-15-0098
122. Boudreau JE, Hsu KC. Natural killer cell education and the response to infection and cancer therapy: stay tuned. *Trends Immunol.* (2018) 39:222–39. doi: 10.1016/j.it.2017.12.001
123. Locatelli F, Pende D, Mingari MC, Bertaina A, Falco M, Moretta A, et al. Cellular and molecular basis of haploidentical hematopoietic stem cell transplantation in the successful treatment of high-risk leukemias: role of alloreactive NK cells. *Front Immunol.* (2013) 4:15. doi: 10.3389/fimmu.2013.00015
124. Joyce MG, Sun PD. The structural basis of ligand recognition by natural killer cell receptors. *J Biomed Biotechnol.* (2011) 2011:203628. doi: 10.1155/2011/203628
125. Misra MK, Augusto DG, Martin GM, Nemat-Gorgani N, Sauter J, Hofmann JA, et al. Report from the Killer-cell Immunoglobulin-like Receptors (KIR) component of the 17th International HLA and Immunogenetics Workshop. *Hum Immunol.* (2018) 79:825–33. doi: 10.1016/j.humimm.2018.10.003
126. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol.* (2002) 169:5118–29. doi: 10.4049/jimmunol.169.9.5118
127. Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev.* (2002) 190:40–52. doi: 10.1034/j.1600-065X.2002.19004.x
128. Jiang W, Johnson C, Jayaraman J, Simecek N, Noble J, Moffatt MF, et al. Copy number variation leads to considerable diversity for B but not A haplotypes of the human KIR genes encoding NK cell receptors. *Genome Res.* (2012) 22:1845–54. doi: 10.1101/gr.137976.112
129. Pyo CW, Guethlein LA, Vu Q, Wang R, Abi-Rached L, Norman PJ, et al. Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS ONE.* (2010) 5:e15115. doi: 10.1371/journal.pone.0015115
130. Leung W, Iyengar R, Triplett B, Turner V, Behm FG, Holladay MS, et al. Comparison of killer Ig-like receptor genotyping and phenotyping for selection of allogeneic blood stem cell donors. *J Immunol.* (2005) 174:6540–5. doi: 10.4049/jimmunol.174.10.6540
131. Leung W. Use of NK cell activity in cure by transplant. *Br J Haematol.* (2011) 155:14–29. doi: 10.1111/j.1365-2141.2011.08823.x
132. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol.* (2011) 32:364–72. doi: 10.1016/j.it.2011.06.001
133. Raulet DH. Missing self recognition and self tolerance of natural killer (NK) cells. *Semin Immunol.* (2006) 18:145–50. doi: 10.1016/j.smim.2006.03.003
134. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity.* (2006) 25:331–42. doi: 10.1016/j.immuni.2006.06.013
135. Orr MT, Murphy WJ, Lanier LL. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol.* (2010) 11:321–7. doi: 10.1038/ni.1849
136. Tarek N, Le Luduec JB, Gallagher MM, Zheng J, Venstrom JM, Chamberlain E, et al. Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *J Clin Invest.* (2012) 122:3260–70. doi: 10.1172/JCI62749
137. Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol.* (2009) 182:4572–80. doi: 10.4049/jimmunol.0803900
138. Boudreau JE, Mulrooney TJ, Le Luduec JB, Barker E, Hsu KC. KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV. *J Immunol.* (2016) 196:3398–410. doi: 10.4049/jimmunol.1502469
139. Valiante NM, Parham P. Natural killer cells, HLA class I molecules, and marrow transplantation. *Biol Blood Marrow Transplant.* (1997) 3:229–35.
140. Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol.* (2004) 172:644–50. doi: 10.4049/jimmunol.172.1.644
141. McQueen KL, Dorighi KM, Guethlein LA, Wong R, Sanjanwala B, Parham P. Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum Immunol.* (2007) 68:309–23. doi: 10.1016/j.humimm.2007.01.019
142. Cooley S, Trachtenberg E, Bergemann TL, Saeteurn K, Klein J, Le CT, et al. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood.* (2009) 113:726–32. doi: 10.1182/blood-2008-07-171926
143. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Le CT, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood.* (2010) 116:2411–9. doi: 10.1182/blood-2010-05-283051
144. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood.* (2007) 110:433–40. doi: 10.1182/blood-2006-07-038687
145. Boudreau JE, Giglio F, Gooley TA, Stevenson PA, Le Luduec JB, Shaffer BC, et al. KIR3DL1/HLA-B subtypes govern acute myelogenous leukemia relapse after hematopoietic cell transplantation. *J Clin Oncol.* (2017) 35:2268–78. doi: 10.1200/JCO.2016.70.7059
146. Cooley S, McCullar V, Wangen R, Bergemann TL, Spellman S, Weisdorf DJ, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. *Blood.* (2005) 106:4370–6. doi: 10.1182/blood-2005-04-1644
147. Mancusi A, Ruggeri L, Urbani E, Pierini A, Massei MS, Carotti A, et al. Haploidentical hematopoietic transplantation from KIR ligand-mismatched donors with activating KIRs reduces nonrelapse mortality. *Blood.* (2015) 125:3173–82. doi: 10.1182/blood-2014-09-599993
148. Oevermann L, Michaelis SU, Mezger M, Lang P, Toporski J, Bertaina A, et al. KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. *Blood.* (2014) 124:2744–7. doi: 10.1182/blood-2014-03-565069
149. Wanquet A, Bramanti S, Harbi S, Furst S, Legrand F, Faucher C, et al. Killer cell immunoglobulin-like receptor-ligand mismatch in donor versus recipient direction provides better graft-versus-tumor effect in patients with hematologic malignancies undergoing allogeneic T cell-replete haploidentical transplantation followed by post-transplant

cyclophosphamide. *Biol Blood Marrow Transplant.* (2018) 24:549–54. doi: 10.1016/j.bbmt.2017.11.042
150. van den Brink MR, Velardi E, Perales MA. Immune reconstitution following stem cell transplantation. *Hematology Am Soc Hematol Educ Program.* (2015) 2015:215–9. doi: 10.1182/asheducation-2015.1.215
151. Ogonek J, Kralj Juric M, Ghimire S, Varanasi PR, Holler E, Greinix H, et al. Immune reconstitution after allogeneic hematopoietic stem cell transplantation. *Front Immunol.* (2016) 7:507. doi: 10.3389/fimmu.2016.00507
152. DeCook LJ, Thoma M, Huneke T, Johnson ND, Wiegand RA, Patnaik MM, et al. Impact of lymphocyte and monocyte recovery on the outcomes of allogeneic hematopoietic SCT with fludarabine and melphalan conditioning. *Bone Marrow Transplantation.* (2012) 48:708. doi: 10.1038/bmt.2012.211
153. Le Bourgeois A, Peterlin P, Guillaume T, Delaunay J, Duquesne A, Le Gouill S, et al. Higher early monocyte and total lymphocyte counts are associated with better overall survival after standard total body irradiation, cyclophosphamide, and fludarabine reduced-intensity conditioning double umbilical cord blood allogeneic stem cell transplantation in adults. *Biol Blood Marrow Transplant.* (2016) 22:1473–9. doi: 10.1016/j.bbmt.2016.04.015
154. Turcotte LM, Cao Q, Cooley SA, Curtsinger J, Holtan SG, Luo X, et al. Monocyte subpopulation recovery as predictors of hematopoietic cell transplantation outcomes. *Biol Blood Marrow Transplant.* (2019) 25:883–90. doi: 10.1016/j.bbmt.2019.01.003
155. Pei X, Zhao X, Wang Y, Xu L, Zhang X, Liu K, et al. Comparison of reference values for immune recovery between event-free patients receiving haploidentical allografts and those receiving human leukocyte antigen-matched sibling donor allografts. *Front Med.* (2018) 12:153–63. doi: 10.1007/s11684-017-0548-1
156. Salzmann-Manrique E, Bremm M, Huenecke S, Stech M, Orth A, Eyrich M, et al. Joint modeling of immune reconstitution post haploidentical stem cell transplantation in pediatric patients with acute leukemia comparing CD34+-selected to CD3/CD19-depleted grafts in a retrospective multicenter study. *Front Immunol.* (2018) 9:1841. doi: 10.3389/fimmu.2018.01841
157. Offner F, Schoch G, Fisher L, Torok-Storb B, Martin P. Mortality hazard functions as related to neutropenia at different times after marrow transplantation. *Blood.* (1996) 88:4058–62. doi: 10.1182/blood.V88.10.4058.bloodjournal88104058
158. Chang Y-J, Zhao X-Y, Huo M-R, Xu L-P, Liu D-H, Liu K-Y, et al. Immune reconstitution following unmanipulated HLA-mismatched/haploidentical transplantation compared with HLA-identical sibling transplantation. *J Clin Immunol.* (2012) 32:268–80. doi: 10.1007/s10875-011-9630-7
159. Lin X, Lu ZG, Song CY, Huang YX, Guo KY, Deng L, et al. Long-term outcome of HLA-haploidentical hematopoietic stem cell transplantation without *in vitro* T-cell depletion based on an FBCA conditioning regimen for hematologic malignancies. *Bone Marrow Transplant.* (2015) 50:1092. doi: 10.1038/bmt.2015.108
160. Fearnley DB, Whyte LF, Carnoutsos SA, Cook AH, Hart DNJ. Monitoring human blood dendritic cell numbers in normal individuals and in stem cell transplantation. *Blood.* (1999) 93:728–36. doi: 10.1182/blood.V93.2.728
161. Soriano-Sarabia N, Sandvold H, Jomaa H, Kubin T, Bein G, Hackstein H. Primary MHC-class II+ cells are necessary to promote resting Vδ2 cell expansion in response to *(E)*-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate and isopentenyl pyrophosphate. *J Immunol.* (2012) 189:5212–22. doi: 10.4049/jimmunol.1200093
162. Wang X, Liu J, Gao H, Mo X-D, Han T, Xu L-P, et al. Dendritic cells are critical for the activation and expansion of Vδ2+ T cells after allogeneic hematopoietic transplantation. *Front Immunol.* (2018) 9:2528. doi: 10.3389/fimmu.2018.02528
163. Roider T, Katzfuß M, Matos C, Singer K, Renner K, Oefner PJ, et al. Antithymocyte globulin induces a tolerogenic phenotype in human dendritic cells. *Int J Mol Sci.* (2016) 17:2081. doi: 10.3390/ijms17122081
164. Monti P, Allavena P, Di Carlo V, Piemonti L. Effects of anti-lymphocytes and anti-thymocytes globulin on human dendritic cells. *Int Immunopharmacol.* (2003) 3:189–96. doi: 10.1016/S1567-5769(02)00253-9
165. Bari R, Rujkijyanont P, Sullivan E, Kang G, Turner V, Gan K, et al. Effect of donor KIR2DL1 allelic polymorphism on the outcome of pediatric allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol.* (2013) 31:3782–90. doi: 10.1200/JCO.2012.47.4007
166. Moretta L, Locatelli F, Pende D, Marcenaro E, Mingari MC, Moretta A. Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *Blood.* (2011) 117:764–71. doi: 10.1182/blood-2010-08-264085
167. Pfeiffer MM, Feuchtinger T, Teltschik HM, Schumm M, Muller I, Handgretinger R, et al. Reconstitution of natural killer cell receptors influences natural killer activity and relapse rate after haploidentical transplantation of T- and B-cell depleted grafts in children. *Haematologica.* (2010) 95:1381–8. doi: 10.3324/haematol.2009.021121
168. Locatelli F, Merli P, Rutella S. At the bedside: innate immunity as an immunotherapy tool for hematological malignancies. *J Leukoc Biol.* (2013) 94:1141–57. doi: 10.1189/jlb.0613343
169. Nguyen S, Dhedin N, Vernant J-P, Kuentz M, Jijakli AA, Rouas-Freiss N, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood.* (2005) 105:4135–42. doi: 10.1182/blood-2004-10-4113
170. Pende D, Marcenaro S, Falco M, Martini S, Bernardo ME, Montagna D, et al. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood.* (2009) 113:3119–29. doi: 10.1182/blood-2008-06-164103
171. Vago L, Forno B, Sormani MP, Crocchiolo R, Zino E, Di Terlizzi S, et al. Temporal, quantitative, and functional characteristics of single-KIR–positive alloreactive natural killer cell recovery account for impaired graft-versus-leukemia activity after haploidentical hematopoietic stem cell transplantation. *Blood.* (2008) 112:3488–99. doi: 10.1182/blood-2007-07-103325
172. Locatelli F, Pende D, Falco M, Della Chiesa M, Moretta A, Moretta L. NK cells mediate a crucial graft-versus-leukemia effect in haploidentical-HSCT to cure high-risk acute leukemia. *Trends Immunol.* (2018) 39:577–90. doi: 10.1016/j.it.2018.04.009
173. Muccio L, Bertaina A, Falco M, Pende D, Meazza R, Lopez-Botet M, et al. Analysis of memory-like natural killer cells in human cytomegalovirus-infected children undergoing alphabeta+T and B cell-depleted hematopoietic stem cell transplantation for hematological malignancies. *Haematologica.* (2016) 101:371–81. doi: 10.3324/haematol.2015.134155
174. Russo A, Oliveira G, Berglund S, Greco R, Gambacorta V, Cieri N, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood.* (2018) 131:247–62. doi: 10.1182/blood-2017-05-780668
175. Roberto A, Di Vito C, Zaghi E, Mazza EMC, Capucetti A, Calvi M, et al. The early expansion of anergic NKG2Apos/CD56dim/CD16neg natural killer represents a therapeutic target in haploidentical hematopoietic stem cell transplantation. *Haematologica.* (2018) 103:1390–402. doi: 10.3324/haematol.2017.186619
176. Zhao X-Y, Huang X-J, Liu K-Y, Xu L-P, Liu D-H. Reconstitution of natural killer cell receptor repertoires after unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation: analyses of CD94:NKG2A and killer immunoglobulin-like receptor expression and their associations with clinical outcome. *Biol. Blood Marrow Transplant.* (2007) 13:734–44. doi: 10.1016/j.bbmt.2007.02.010
177. Hu L-J, Zhao X-Y, Yu X-X, Lv M, Han T-T, Han W, et al. Quantity and quality reconstitution of NKG2A+ natural killer cells are associated with graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* (2019) 25:1–11. doi: 10.1016/j.bbmt.2018.08.008
178. Zhao X-y, Huang X-j, Liu K-y, Xu L-p, Liu D-h. Prognosis after unmanipulated HLA-haploidentical blood and marrow transplantation is correlated to the numbers of KIR ligands in recipients. *Eur J Haematol.* (2007) 78:338–46. doi: 10.1111/j.1600-0609.2007.00822.x
179. Raiola A, Dominietto A, Varaldo R, Ghiso A, Galaverna F, Bramanti S, et al. Unmanipulated haploidentical BMT following non-myeloablative conditioning and post-transplantation CY for advanced Hodgkin's lymphoma. *Bone Marrow Transplant.* (2013) 49:190. doi: 10.1016/j.bbmt.2012.08.014
180. Cieri N, Greco R, Crucitti L, Morelli M, Giglio F, Levati G, et al. Post-transplantation cyclophosphamide and sirolimus after haploidentical hematopoietic stem cell transplantation using a treosulfan-based

myeloablative conditioning and peripheral blood stem cells. *Biol Blood Marrow Transplant.* (2015) 21:1506–14. doi: 10.1016/j.bbmt.2015.04.025
181. McCurdy SR, Vulic A, Symons HJ, Towlerton AM, Valdez BC, Jones RJ, et al. Comparable and robust immune reconstitution after HLA-haploidentical or HLA-matched allogeneic transplantation (BMT) utilizing posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2015) 21:S71. doi: 10.1016/j.bbmt.2014.11.075
182. Roberto A, Castagna L, Zanon V, Bramanti S, Crocchiolo R, McLaren JE, et al. Role of naive-derived T memory stem cells in T-cell reconstitution following allogeneic transplantation. *Blood.* (2015) 125:2855–64. doi: 10.1182/blood-2014-11-608406
183. McCurdy SR, Luznik L. Immune reconstitution after T-cell replete HLA-haploidentical transplantation. *Semin Hematol.* (2019) 56:221–6. doi: 10.1053/j.seminhematol.2019.03.005
184. Tian D-M, Wang Y, Zhang X-H, Liu K-Y, Huang X-J, Chang Y-J. Rapid recovery of CD3+CD8+ T cells on day 90 predicts superior survival after unmanipulated haploidentical blood and Marrow transplantation. *PLoS ONE.* (2016) 11:e0156777. doi: 10.1371/journal.pone.0156777
185. Kim DH, Sohn SK, Won DI, Lee NY, Suh JS, Lee KB. Rapid helper T-cell recovery above 200 × 106/l at 3 months correlates to successful transplant outcomes after allogeneic stem cell transplantation. *Bone Marrow Transplant.* (2006) 37:1119–28. doi: 10.1038/sj.bmt.1705381
186. Bondanza A, Ruggeri L, Noviello M, Eikema D-J, Bonini C, Chabannon C, et al. Beneficial role of CD8+ T-cell reconstitution after HLA-haploidentical stem cell transplantation for high-risk acute leukaemias: results from a clinico-biological EBMT registry study mostly in the T-cell-depleted setting. *Bone Marrow Transplant.* (2019) 54:867–76. doi: 10.1038/s41409-018-0351-x
187. Cieri N, Oliveira G, Greco R, Forcato M, Taccioli C, Cianciotti B, et al. Generation of human memory stem T cells after haploidentical T-replete hematopoietic stem cell transplantation. *Blood.* (2015) 125:2865–74. doi: 10.1182/blood-2014-11-608539
188. Fisher SA, Lamikanra A, Dorée C, Gration B, Tsang P, Danby RD, et al. Increased regulatory T cell graft content is associated with improved outcome in haematopoietic stem cell transplantation: a systematic review. *Brit J Haematol.* (2017) 176:448–63. doi: 10.1111/bjh.14433
189. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+ CD4+ CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood.* (2005) 106:2903–11. doi: 10.1182/blood-2005-03-1257
190. Peccatori J, Forcina A, Clerici D, Crocchiolo R, Vago L, Stanghellini MT, et al. Sirolimus-based graft-versus-host disease prophylaxis promotes the *in vivo* expansion of regulatory T cells and permits peripheral blood stem cell transplantation from haploidentical donors. *Leukemia.* (2015) 29:396–405. doi: 10.1038/leu.2014.180
191. Wang Y, Zhao XY, Xu LP, Zhang XH, Han W, Chen H, et al. Lower incidence of acute GVHD is associated with the rapid recovery of CD4(+)CD25(+)CD45RA(+) regulatory T cells in patients who received haploidentical allografts from NIMA-mismatched donors: a retrospective (development) and prospective (validation) cohort-based study. *Oncoimmunology.* (2016) 5:e1242546. doi: 10.1080/2162402X.2016.1242546
192. Vantourout P, Hayday A. Six-of-the-best: unique contributions of γδ T cells to immunology. *Nat Rev Immunol.* (2013) 13:88. doi: 10.1038/nri3384
193. Kabelitz D, Kalyan S, Oberg H-H, Wesch D. Human Vδ2 versus non-Vδ2 γδ T cells in antitumor immunity. *OncoImmunology.* (2013) 2:e23304. doi: 10.4161/onci.23304
194. Knight A, Madrigal AJ, Grace S, Sivakumaran J, Kottaridis P, Mackinnon S, et al. The role of Vδ2-negative γδ T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. *Blood.* (2010) 116:2164–72. doi: 10.1182/blood-2010-01-255166
195. Storek J, Geddes M, Khan F, Huard B, Helg C, Chalandon Y, et al. Reconstitution of the immune system after hematopoietic stem cell transplantation in humans. *Semin Immunopathol.* (2008) 30:425. doi: 10.1007/s00281-008-0132-5
196. Roberto A, Castagna L, Gandolfi S, Zanon V, Bramanti S, Sarina B, et al. B-cell reconstitution recapitulates B-cell lymphopoiesis following haploidentical BM transplantation and post-transplant CY. *Bone Marrow Transplant.* (2014) 50:317. doi: 10.1038/bmt.2014.266
197. Vago L, Perna SK, Zanussi M, Mazzi B, Barlassina C, Stanghellini MT, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med.* (2009) 361:478–88. doi: 10.1056/NEJMoa0811036
198. Ahci M, Stempelmann K, Buttkereit U, Crivello P, Trilling M, Heinold A, et al. Clinical utility of quantitative PCR for chimerism and engraftment monitoring after allogeneic stem cell transplantation for hematologic malignancies. *Biol Blood Marrow Transplant.* (2017) 23:1658–68. doi: 10.1016/j.bbmt.2017.05.031
199. Crucitti L, Crocchiolo R, Toffalori C, Mazzi B, Greco R, Signori A, et al. Incidence, risk factors and clinical outcome of leukemia relapses with loss of the mismatched HLA after partially incompatible hematopoietic stem cell transplantation. *Leukemia.* (2015) 29:1143–52. doi: 10.1038/leu.2014.314
200. McCurdy SR, Iglehart BS, Batista DA, Gocke CD, Ning Y, Knaus HA, et al. Loss of the mismatched human leukocyte antigen haplotype in two acute myelogenous leukemia relapses after haploidentical bone marrow transplantation with post-transplantation cyclophosphamide. *Leukemia.* (2016) 30:2102–6. doi: 10.1038/leu.2016.144
201. Toffalori C, Zito L, Gambacorta V, Riba M, Oliveira G, Bucci G, et al. Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nat Med.* (2019) 25:603–11. doi: 10.1038/s41591-019-0400-z
202. Noviello M, Manfredi F, Ruggiero E, Perini T, Oliveira G, Cortesi F, et al. Bone marrow central memory and memory stem T-cell exhaustion in AML patients relapsing after HSCT. *Nat Commun.* (2019) 10:1065. doi: 10.1038/s41467-019-08871-1
203. Khan MA, Bashir Q, Chaudhry QU, Ahmed P, Satti TM, Mahmood SK. Review of haploidentical hematopoietic cell transplantation. *J Glob Oncol.* (2018) 4:1–13. doi: 10.1200/JGO.18.00130
204. Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol.* (2009) 10:489–500. doi: 10.1016/S1470-2045(09)70074-9
205. Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science.* (1997) 276:1719–24. doi: 10.1126/science.276.5319.1719
206. Vago L, Oliveira G, Bondanza A, Noviello M, Soldati C, Ghio D, et al. T-cell suicide gene therapy prompts thymic renewal in adults after hematopoietic stem cell transplantation. *Blood.* (2012) 120:1820–30. doi: 10.1182/blood-2012-01-405670
207. Straathof KC, Pule MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. An inducible caspase 9 safety switch for T-cell therapy. *Blood.* (2005) 105:4247–54. doi: 10.1182/blood-2004-11-4564
208. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med.* (2011) 365:1673–83. doi: 10.1056/NEJMoa1106152
209. Zhou X, Dotti G, Krance RA, Martinez CA, Naik S, Kamble RT, et al. Inducible caspase-9 suicide gene controls adverse effects from alloreplete T cells after haploidentical stem cell transplantation. *Blood.* (2015) 125:4103–13. doi: 10.1182/blood-2015-02-628354
210. Zhou X, Di Stasi A, Tey SK, Krance RA, Martinez C, Leung KS, et al. Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood.* (2014) 123:3895–905. doi: 10.1182/blood-2014-01-551671
211. Zeidan AM, Forde PM, Symons H, Chen A, Smith BD, Pratz K, et al. HLA-haploidentical donor lymphocyte infusions for patients with relapsed hematologic malignancies after related HLA-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant.* (2014) 20:314–8. doi: 10.1016/j.bbmt.2013.11.020
212. Sun W, Mo XD, Zhang XH, Xu LP, Wang Y, Yan CH, et al. Chemotherapy plus DLI for relapse after haploidentical HSCT: the biological characteristics of relapse influences clinical outcomes of acute leukemia patients. *Bone Marrow Transplant.* (2018) 54:1198–207. doi: 10.1038/s41409-018-0406-z
213. Gao XN, Lin J, Wang SH, Huang WR, Li F, Li HH, et al. Donor lymphocyte infusion for prevention of relapse after unmanipulated haploidentical PBSCT for very high-risk hematologic malignancies. *Ann Hematol.* (2019) 98:185–93. doi: 10.1007/s00277-018-3482-7

214. Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, et al. Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T cells. *Front Immunol.* (2014) 5:16. doi: 10.3389/fimmu.2014.00016
215. Muller N, Landwehr K, Langeveld K, Stenzel J, Pouwels W, van der Hoorn M, et al. Generation of alloreactivity-reduced donor lymphocyte products retaining memory function by fully automatic depletion of CD45RA-positive cells. *Cytotherapy.* (2018) 20:532–42. doi: 10.1016/j.jcyt.2018 01.006
216. Schroeder T, Rachlis E, Bug G, Stelljes M, Klein S, Steckel NK, et al. Treatment of acute myeloid leukemia or myelodysplastic syndrome relapse after allogeneic stem cell transplantation with azacitidine and donor lymphocyte infusions–a retrospective multicenter analysis from the German Cooperative Transplant Study Group. *Biol Blood Marrow Transplant.* (2015) 21:653–60. doi: 10.1016/j.bbmt.2014.12.016
217. Schroeder T, Rautenberg C, Kruger W, Platzbecker U, Bug G, Steinmann J, et al. Treatment of relapsed AML and MDS after allogeneic stem cell transplantation with decitabine and DLI-a retrospective multicenter analysis on behalf of the German Cooperative Transplant Study Group. *Ann Hematol.* (2018) 97:335–42. doi: 10.1007/s00277-017-3185-5
218. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med.* (2014) 371:1507–17. doi: 10.1056/NEJMoa1407222
219. Gardner RA, Finney O, Annesley C, Brakke H, Summers C, Leger K, et al. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. *Blood.* (2017) 129:3322–31. doi: 10.1182/blood-2017-02-769208
220. Locke FL, Ghobadi A, Jacobson CA, Miklos DB, Lekakis LJ, Oluwole OO, et al. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. *Lancet Oncol.* (2019) 20:31–42. doi: 10.1016/S1470-2045(18) 30864-7
221. Locke FL, Neelapu SS, Bartlett NL, Siddiqi T, Chavez JC, Hosing CM, et al. Phase 1 results of ZUMA-1: a multicenter study of KTE-C19 anti-CD19 CAR T cell therapy in refractory aggressive lymphoma. *Mol Ther.* (2017) 25:285–95. doi: 10.1016/j.ymthe.2016.10.020
222. Qasim W, Zhan H, Samarasinghe S, Adams S, Amrolia P, Stafford S, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med.* (2017) 9:eaaj2013. doi: 10.1126/scitranslmed.aaj2013
223. Tang X, Yang L, Li Z, Nalin AP, Dai H, Xu T, et al. First-in-man clinical trial of CAR NK-92 cells: safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. *Am J Cancer Res.* (2018) 8:1083–9.
224. Raje N, Berdeja J, Lin Y, Siegel D, Jagannath S, Madduri D, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. *N Engl J Med.* (2019) 380:1726–37. doi: 10.1056/NEJMoa1817226
225. Baumeister SH, Murad J, Werner L, Daley H, Trebeden-Negre H, Gicobi JK, et al. Phase I trial of autologous CAR T cells targeting NKG2D ligands in patients with AML/MDS and multiple myeloma. *Cancer Immunol Res.* (2019) 7:100–12. doi: 10.1158/2326-6066.CIR-18-0307
226. Kim MY, Yu KR, Kenderian SS, Ruella M, Chen S, Shin TH, et al. Genetic inactivation of CD33 in hematopoietic stem cells to enable CAR T cell immunotherapy for acute myeloid leukemia. *Cell.* (2018) 173:1439–53.e19. doi: 10.1016/j.cell.2018.05.013
227. Pizzitola I, Anjos-Afonso F, Rouault-Pierre K, Lassailly F, Tettamanti S, Spinelli O, et al. Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells *in vivo*. *Leukemia.* (2014) 28:1596–605. doi: 10.1038/leu.2014.62
228. Copelan OR, Sanikommu SR, Trivedi JS, Butler C, Ai J, Ragon BK, et al. Higher incidence of hemorrhagic cystitis following haploidentical related donor transplantation compared with matched related donor transplantation. *Biol Blood Marrow Transplant.* (2019) 25:785–90. doi: 10.1016/j.bbmt.2018.12.142
229. Leen AM, Christin A, Myers GD, Liu H, Cruz CR, Hanley PJ, et al. Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. *Blood.* (2009) 114:4283–92. doi: 10.1182/blood-2009-07-232454
230. Tzannou I, Papadopoulou A, Naik S, Leung K, Martinez CA, Ramos CA, et al. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, epstein-barr virus, and adenovirus infections after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol.* (2017) 35:3547–57. doi: 10.1200/JCO.2017.73.0655
231. Chapuis AG, Egan DN, Bar M, Schmitt TM, McAfee MS, Paulson KG, et al. T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. *Nat Med.* (2019) 25:1064–72. doi: 10.1038/s41591-019-0472-9
232. Marijt WA, Heemskerk MH, Kloosterboer FM, Goulmy E, Kester MG, van der Hoorn MA, et al. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci USA.* (2003) 100:2742–7. doi: 10.1073/pnas.0530192100
233. Dossa RG, Cunningham T, Sommermeyer D, Medina-Rodriguez I, Biernacki MA, Foster K, et al. Development of T-cell immunotherapy for hematopoietic stem cell transplantation recipients at risk of leukemia relapse. *Blood.* (2018) 131:108–20. doi: 10.1182/blood-2017-07-791608
234. Cooley S, Parham P, Miller JS. Strategies to activate NK cells to prevent relapse and induce remission following hematopoietic stem cell transplantation. *Blood.* (2018) 131:1053–62. doi: 10.1182/blood-2017-08-752170
235. Ciurea SO, Schafer JR, Bassett R, Denman CJ, Cao K, Willis D, et al. Phase 1 clinical trial using mbIL21 *ex vivo*-expanded donor-derived NK cells after haploidentical transplantation. *Blood.* (2017) 130:1857–68. doi: 10.1182/blood-2017-05-785659
236. Moon BI, Kim TH, Seoh JY. Functional modulation of regulatory T cells by IL-2. *PLoS ONE.* (2015) 10:e0141864. doi: 10.1371/journal.pone.0141864
237. Bachanova V, Cooley S, Defor TE, Verneris MR, Zhang B, McKenna DH, et al. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood.* (2014) 123:3855–63. doi: 10.1182/blood-2013-10-532531
238. Cooley S, He F, Bachanova V, Vercellotti GM, DeFor TE, Curtsinger JM, et al. First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia. *Blood Adv.* (2019) 3:1970–80. doi: 10.1182/bloodadvances.2018028332
239. Romee R, Rosario M, Berrien-Elliott MM, Wagner JA, Jewell BA, Schappe T, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med.* (2016) 8:357ra123. doi: 10.1126/scitranslmed.aaf2341
240. Romee R, Schneider SE, Leong JW, Chase JM, Keppel CR, Sullivan RP, et al. Cytokine activation induces human memory-like NK cells. *Blood.* (2012) 120:4751–60. doi: 10.1182/blood-2012-04-419283
241. Romee R, Cooley S, Berrien-Elliott MM, Westervelt P, Verneris MR, Wagner JE, et al. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. *Blood.* (2018) 131:2515–27. doi: 10.1182/blood-2017-12-823757

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REVIEW
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Mechanisms of Leukemia Immune Evasion and Their Role in Relapse After Haploidentical Hematopoietic Cell Transplantation

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Over the last decade, the development of multiple strategies to allow the safe transfer from the donor to the patient of high numbers of partially HLA-incompatible T cells has dramatically reduced the toxicities of haploidentical hematopoietic cell transplantation (haplo-HCT), but this was not accompanied by a similar positive impact on the incidence of post-transplantation relapse. In the present review, we will elaborate on how the unique interplay between HLA-mismatched immune system and malignancy that characterizes haplo-HCT may impact relapse biology, shaping the selection of disease variants that are resistant to the "graft-vs.-leukemia" effect. In particular, we will present current knowledge on genomic loss of HLA, a relapse modality first described in haplo-HCT and accounting for a significant proportion of relapses in this setting, and discuss other more recently identified mechanisms of post-transplantation immune evasion and relapse, including the transcriptional downregulation of HLA class II molecules and the enforcement of inhibitory checkpoints between T cells and leukemia. Ultimately, we will review the available treatment options for patients who relapse after haplo-HCT and discuss on how a deeper insight into relapse immunobiology might inform the rational and personalized selection of therapies to improve the largely unsatisfactory clinical outcome of relapsing patients.

Keywords: haploidentical allogeneic hematopoietic stem cell transplantation, relapse, immune escape, HLA, immune check point

INTRODUCTION

Allogeneic hematopoietic cell transplantation from haploidentical family members represents a promising solution to offer allogeneic HCT to virtually all patients with an indication to transplant, but lacking a fully compatible and/or rapidly available donor. However, from the immunological standpoint, haplo-HCT also represents the most challenging transplantation setting, counterpoising two largely HLA-incompatible immune systems and thus posing a severe risk of graft-vs.-host disease (GvHD) and immune rejection. To overcome this obstacle, over the last few decades, many strategies have been developed to improve the feasibility and safety of haplo-HCT (1, 2). In particular, two main haplo-HCT "philosophies" were progressively refined over

the years: the *ex vivo* manipulation of the graft to deplete the most alloreactive cell subsets (3), eventually reinfusing them in a subsequent moment in combination with regulatory T cells (4, 5) or upon incorporation of safety switches (6–8), vs. the infusion of unmanipulated grafts, followed by administration of drugs capable of eliminating alloreactive cells *in vivo* (9, 10). Noticeably, some of these platforms have demonstrated remarkable success, leading to an exponential increase in the number of haplo-HCT performed worldwide (11, 12).

The development of innovative strategies to render haplo-HCT feasible was fueled by intensive research on the immunobiology of allo-HCT, leading to a number of observations that were later extended to other transplantation settings or even served as the foundation to explain the physiological metrics of immune responses to pathogens and tumors.

In the present review, we will present one of the most paradigmatic examples of this process by describing how investigation of mechanisms of relapse after haplo-HCT paved the way to understanding the interplay between transplanted immune system and tumor also in other transplantation settings and, importantly, to the development of new rationales for relapse therapy.

TUMOR-INTRINSIC MECHANISMS OF RELAPSE

Seminal studies conducted by the Seattle group more than 25 years ago led to the identification of donor-derived T cells as one of the major drivers of the graft-vs.-leukemia (GvL) effect (13). It is thus no surprise that all the best-characterized tumor-intrinsic mechanisms of immune evasion and relapse after allo-HCT have as a final output the abrogation of interactions between T cells and the tumor. This can occur either because leukemia cells become "invisible" to patrolling T cells, for instance through genetic or epigenetic alterations in the antigen processing and presenting machinery, or because they enact mechanisms to render the encounter ineffectual, as when inhibitory immune checkpoints are enforced (**Figure 1**).

Genomic Loss of HLA

Alterations in the expression and functionality of HLA class I and II molecules have long been characterized in solid tumors, underlining also in this setting the importance of T cell-mediated responses in shaping tumor immunogenicity (14).

Interestingly, in hematological tumors, and acute myeloid leukemia (AML) in particular, alterations in the HLA region are quite uncommon, especially at the time of diagnosis (15, 16). This feature is critical, since the donor T cell-mediated GvL effect of allo-HCT mostly depends on the HLA molecule expression on the surface of leukemic cells. As part of the antigen-presenting machinery, HLA molecules serve as restriction elements for minor histocompatibility antigens and tumor-associated antigens or, when incompatible, as direct targets of primary alloreactivity. In haplo-HCT especially, where an entire HLA haplotype is mismatched between patient and donor, T cell-mediated alloreactivity converges against the incompatible molecules that rapidly become the immunodominant GvL targets.

Given this fundamental role of HLAs in the biology of haplo-HCT, it is reasonable that a possible getaway for malignant cells to escape the bottleneck of immunological pressure might be to exploit alterations in the HLA locus, mirroring what happens in solid tumors.

The first characterization of such a strategy being used in AML after haplo-HCT was provided nearly 10 years ago, when genomic loss of the mismatched HLA haplotype (from this point on referred to as "HLA loss") was first reported (17). Behind this discovery, there is a curious case of serendipity: While investigating intermediate-resolution genomic HLA typing of bone marrow aspirate samples as an alternative technique for the assessment of hematopoietic chimerism (18), our group encountered several cases of AML post-transplantation relapse that typed negative for the patient HLAs. Genomic HLA typing of leukemic blasts purified from these relapses confirmed the absence of all HLA class I and class II genes encoded on the mismatched patient-specific HLA haplotype. A deeper examination of this phenomenon was then carried out exploiting whole-genome single-nucleotide polymorphism (SNP) arrays, demonstrating loss of heterozygosity (LOH) of chromosome 6p in the absence of copy number variations (CNVs), thus suggesting an event of acquired somatic uniparental disomy (aUPD). UPD has been described as a common chromosomal aberration in different tumor types, both solid and hematological (19–21). This genomic alteration consists of the loss of a chromosome region that is subsequently replaced by the homologous copy, resulting in acquired homozygosity of that region without the actual loss of genomic material. The consequences of this event can be diverse: We can witness an increase in the expression of oncogenes, loss of heterozygosity of mutated tumor-suppressors, or in this specific context, the loss of the HLA molecules not shared between donor and recipient, which represented the most immunodominant targets for donor T cell alloreactivity. In the case of HLA loss, the observed rearrangements had variable boundaries and extension in the different patients, but in most cases, encompassed the entire HLA region and, therefore, included all HLA class I and class II loci.

Ex vivo coculture of donor T cells with leukemic cells demonstrated that when HLA loss occurs, mutated blasts become completely invisible to donor T cells that were capable of recognizing them before transplantation, thus taking the upper hand over other clones and rapidly becoming the predominant population (17, 22). Documentation of HLA loss not only provides an explanation for how disease escaped a pre-existing control, but also contraindicates the infusion of additional donor T cells as a strategy to try to revert relapse, since also these cells would fail to find a target to attack.

Conversely, HLA loss variants that become invisible to donor T cell allorecognition could in principle still represent viable targets for alloreactive donor natural killer (NK) cells. Indeed, while the mechanism of aUPD does not reduce the overall surface levels of HLA class I molecules on the leukemia cell surface, thus avoiding to trigger "missing self" recognition by NK cells (23),

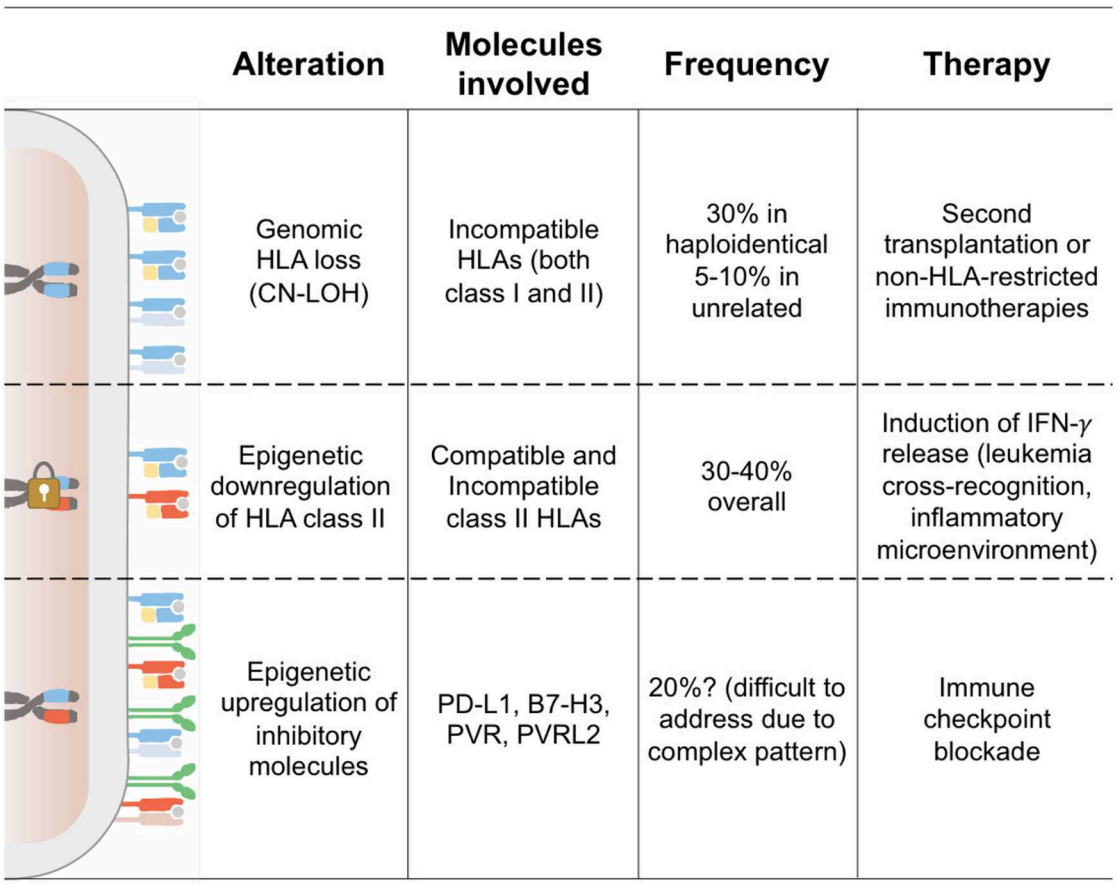

FIGURE 1 | Tumor-Intrinsic Mechanisms of Immune Evasion and Relapse. This cartoon summarizes the features of the three modalities of leukemia immune evasion and relapse after allo-HCT better characterized to date. Chromosomes indicate the HLA haplotype homo- or hetero-zygosity, showing in cyan the donor-recipient shared haplotype and in red the patient-specific incompatible haplotype. The padlock symbolizes epigenetic silencing of the HLA class II loci. On the cell surface, HLA class I molecules are shown as heterodimers of HLA and beta-2-microglobulin (in yellow), HLA class II as dimers of two transmembrane single-chain HLA molecules, and inhibitory ligands as green homodimers.

the HLA alleles that are lost by leukemia cells often also represent ligands for donor inhibitory KIRs (24). Nonetheless, HLA loss relapses still occur, and the biology at the basis of NK cell failure in preventing or controlling the emergence of HLA loss relapses needs to be investigated further. This is highly relevant from the translational standpoint, since an improved understanding of NK cell responses in the context of HLA loss could also serve as a springboard to design adoptive immunotherapy trials based on NK cells to treat, or even prevent, these relapse variants.

One of the most relevant open issues regarding HLA loss is understanding when the genetic alteration occurs, or in other terms, if an infinitesimally small immune-resistant clone exists before allo-HCT or not. To date, the molecular drivers of aUPD are poorly known. It has been demonstrated that an increased susceptibility for chromosomal breaks and the effects of DNA damage inducers, including chemotherapeutic agents, might lead to higher aUPD risk in tight proximity of mitotic recombination sites (20), jeopardizing those heavily treated patients who undergo the transplantation procedure after multiple lines of chemotherapy. However, there is also evidence that aUPD can also be a common finding in AML samples at the time of diagnosis, with a large study on 454 samples reporting aUPD frequency of 15–20%. This alteration mainly affects specific chromosome arms, including 13q, 11p, and 11q (25). Of note in these reports, the involvement of the HLA region located in chromosome 6p is exceptional, with an estimated 3–4% of myeloid malignancies characterized by HLA LOH at disease onset (26, 27).

Some suggestions on the biological origin of HLA loss relapses come from retrospective clinical studies. In the largest analysis on this topic performed to date (28), HLA loss relapses were shown to occur significantly later than their "classical" counterparts and to be strongly associated to allo-HCTs performed in an active disease stage. A possible explanation linking these two observations might be that patients transplanted with a sizable leukemia burden probably present also much higher intratumoral heterogeneity than those transplanted with minimal or even undetectable residual disease and are thus also more likely to carry a clone with HLA loss or with high predisposition to aUPD, which may then slowly but steadily grow in the subsequent months following transplantation.

Soon after the initial description (17), a number of other studies reported cases of HLA loss relapses after haplo-HCT, with an incidence ranging between 20 and 40% of all relapses occurring in this setting (22, 29, 30). Of interest, analysis of two different cohorts transplanted at our institution using the same haplo-HCT backbone and differing only in the use of anti-thymocyte globulin (31) or high-dose cyclophosphamide (32) as *in vivo* T cell-depleting agent showed superimposable frequency of HLA loss relapses, suggesting that regardless of the strategy used, a significant population of alloreactive T cells escapes the initial purging and is capable to mediate significant antileukemic immune pressure. Studies specifically focused on T cell-depleted haplo-HCTs are to date lacking, but available data from T cell replete platforms indicate that the frequency of HLA loss is directly associated to the number of T cells transferred as part of the graft or after that (28), thus suggesting that in "T cell naked" transplants, HLA loss might be a rare event and relapses might have different underlying biology.

Interestingly, there have been reports of HLA loss relapses occurring in other transplantation settings, in particular after mismatched unrelated donor HCT (33–36) and, less frequently, after matched unrelated donor HCT (36). Although these reports originate from small cohorts of patients and therefore cannot provide an accurate estimate of the actual incidence of HLA loss in these settings, they appear to indicate a lower incidence of the phenomenon as compared to haplo-HCT. We can speculate that this lower frequency might indicate that, when donor-recipient incompatibilities are fewer, T cell alloreactivity and GvL effect is less pronouncedly focused against incompatible HLAs and possibly outperformed by immunodominant minor histocompatibility antigens. Moreover, it should be considered that in the unrelated HCT setting, the incompatibilities are often not *in cis* on the same haplotype, meaning that losing one haplotype by aUPD might not be as effective in abrogating immune pressure as in the haplo-HCT setting. Finally, the relative contribution of mismatches at the different HLA loci in driving alloreactivity and the GvL effect is not entirely clear, and it may be possible that some, but not other, incompatibilities might be more potent in promoting immune escape by HLA loss. In conclusion, more studies regarding the characterization of relapses outside of the haploidentical setting are still needed, and the complete understanding of how the GvL effect and the strength of the selective pressure mediated by alloreactive T-cells influences and shapes the underlying biological mechanisms of relapse in these contexts is yet to be entirely dissected.

It has already been stated how the occurrence of this genomic alteration greatly impairs T cell allorecognition, prompting the need for a more personalized clinical management of these relapses. As a consequence, the acute leukemia working party (ALWP) of the European Society for Blood and Marrow Transplantation (EBMT) recently made recommendations for testing eventual HLA loss at the time of relapse before employing donor lymphocyte infusions (DLIs) (37). However, until recently, documentation of HLA loss at relapse required the presence of a considerable tumor burden to perform HLA typing of either unprocessed bone marrow samples or, when possible, sorted leukemic blasts (18). To overcome these limitations, we recently developed "HLA-KMR," a rapid, reliable, and economic assay based on quantitative PCR (qPCR) (38) that almost immediately became a commercially available diagnostic tool (GenDx, The Netherlands). The rationale of HLA-KMRs is to combine the detection of non-HLA-polymorphisms together with *ad hoc* qPCR reactions targeting the most common HLA allele groups. Therefore, in "classical" relapses, non-HLA and patient-specific HLA markers are concordantly positive, whereas the absence of HLA-specific signal indicates HLA loss relapse. This tool provides a sensitive method to detect HLA loss relapses event at early stages, allowing fast clinical decision-making and the use of a personalized therapeutic approach for every patient.

Finally, what should be the most appropriate therapeutic approach for HLA loss relapses occurring after haplo-HCT? Taking into consideration the mechanism and immunological consequences of this genomic alteration, a possible strategy could be a second haploidentical transplantation from an alternative donor, selected to target the remaining HLA haplotype. This originates from a unique situation, where donor T cells still share one haplotype with non-hematopoietic tissues, while being fully mismatched with the leukemic blasts, possibly providing an even stronger GvL effect (39). As a proof of concept, this approach was the one associated with the longest survival after relapse for patients experiencing HLA loss at our center (28) and might explain the superior outcome described by Imus and collaborators upon choosing donors with a different HLA-haplotype for second haplo-HCT (40). Unfortunately, a second allo-HCT is often not feasible in elderly or heavily pretreated patients, prompting further preclinical and clinical studies to treat HLA loss relapses using non-HLA-restricted immunotherapy approaches, including bispecific antibodies and chimeric antigen receptor (CAR)-modified T cells.

Downregulation of HLA Class II Molecules

Two very recent studies provided remarkable evidence that genomic haplotype loss is not the only strategy used by leukemic cells to alter their HLA assets and avoid detection by donor-derived T cells. In both studies, comparison of samples pairwise collected from patients before and after allo-HCT led to appreciate that in up to 40% of post-transplantation relapses, the surface expression of HLA class II molecules (HLA-DR, -DQ, and -DP) becomes virtually absent, and this translates to the failure of donor T cells primed against the original disease to recognize the relapse variants (41, 42). Supporting previous studies conducted in animal models (43), this evidence suggests that interactions between HLA class II molecules and CD4 T cells are necessary for a proficient GvL effect and that this non-redundant arm of the antitumor immunity represents a vulnerability that is easily exploited by leukemia to reemerge. It should be noted, however, that HLA class II expression is also emerging as a relevant prognostic parameter in a number of other malignancies. HLA class II negativity has in fact been linked to unfavorable outcome in patients diagnosed with germinal center B-cell like diffuse large B cell lymphoma (44, 45) and with microsatellite stable carcinomas (46). In a study performed on relapsed/refractory classical Hodgkin's lymphoma, in addition to the positivity for PD-L1, high surface expression of HLA class II molecules also

correlated with a better response to the anti PD-1 monoclonal antibody nivolumab (47).

Coming back to leukemia post-transplantation relapses, similar to the previously described genomic HLA loss mechanism, in this case a higher dose of T cells infused with the graft is also associated with a higher likelihood of also experiencing this modality of relapse (41). However, different from haplotype loss, class II downregulation has to date been observed with similar frequencies in both HLA-compatible and incompatible transplants (41, 42). This observation suggests that the driver of this event might not be alloreactivity toward incompatible HLA class II molecules, but rather against their presented repertoire of tumor-specific antigens and minor histocompatibility antigens. Recently, *in silico* studies convincingly showed that the number of minor histocompatibility antigens that are presented by HLA class II molecules surpasses its HLA class I counterpart by more than one logarithm (48, 49), suggesting that in the unrelated donor setting, immune reactivity against minors might be even more potent than the one against the few incompatible HLA molecules.

Of note, in both studies that first described HLA class II downregulation as an immune escape modality, in-depth genetic profiling of the relapsed leukemia found no evidence of mutations in HLA genes or their regulators, arguing toward an epigenetic origin of the observed phenotype. Gene expression analysis, performed to assess the mechanism of HLA class II expression defects, revealed a significant downregulation of the major histocompatibility class II transactivator CIITA (*MHC2TA*) (41, 42), which in some patients was linked to hypermethylation of its promoters (42). We further showed that this feature is stably maintained upon transplantation and serial passages in immune-compromised mice, with levels of surface expression of HLA class II molecules in patient-derived xenografts (PDXs) perfectly mirroring those observed in the corresponding primary human samples (41).

Unexpectedly, however, when we infused donor-derived T cells to animals harboring the HLA class II-expressing diagnosis or the HLA class II-defective relapse, we observed that, although with a slower kinetics, the latter was also eventually recognized and eradicated. An in-depth study of this phenomenon showed that cross-recognition of murine antigens by the infused T cells led to the release of high levels of interferon-γ (IFN-γ) in the animal plasma and that this was followed by the recovery of HLA class II expression on leukemic cells (41). These findings were also confirmed by *ex vivo* experiments, in which post-transplantation leukemic blasts exposed to recombinant human IFN-γ recovered HLA class II expression, and this in turn reconvened donor T cell-mediated recognition (41, 42). From a translational perspective, these results imply that a proinflammatory environment, driven by GvHD or recognition of antigens presented by HLA class I molecules, might actually revert this mechanism of relapse and re-establish a proficient antileukemic response.

Whereas, the description of deregulated HLA class II expression as a mechanism of AML post-transplantation relapse is extremely recent, there are a number of other malignancies in which alterations in HLA class II have been extensively investigated and that might provide precious hints on the molecular driver of the phenomenon in AML. For instance, there have been several reports of HLA class II downregulation in lymphoma cells as a consequence of deletions and point mutations of HLA class II genes and their regulators, including CIITA (44, 45). Moreover, in lymphomas, CIITA has been reported to be a recurrent fusion partner of the programmed death-ligands *CD274/PD-L1* and *CD273/PD-L2*, leading to the downregulation of HLA class II genes and the upregulation of PD-L1 and PD-L2 (50). In addition, loss of HLA class II expression has also been linked to epigenetic silencing, as a consequence of mutations in epigenetic regulators (e.g., enhancer of zeste homolog 2, *EZH2*) or of hypermethylation or hypoacetylation of the promoters of HLA genes and/or CIITA (42, 44, 45, 51). Finally, Tarafdar et al. also proposed a cytokine-mediated pathway of HLA class II silencing active in chronic myeloid leukemia: In this disease, tumor cells can produce anti-inflammatory cytokines including IL-4 (52) and TGF-β (53) that downregulate the expression of CIITA, rendering themselves less immunogenic and susceptible to T cell recognition (52, 54).

Upregulation of T Cell Inhibitory Ligands

While genomic and epigenetic alterations in HLA genes have all the final effect of turning tumor cells invisible to the donor-derived immune system, there is emerging evidence that leukemic cells can also hide in plain sight, using their encounter with T cells to transmit inhibitory signals that stun and impair antigen-specific responses. A number of reports have in fact shown that over the course of treatments and in particular after allo-HCT, hematologic malignancies increase their expression of molecules that inhibit T cell responses or drive their exhaustion, including members of the programmed death-ligand family (41, 55). In a recent study, retrospectively analyzing samples pairwise collected from AML patients at the time of diagnosis and at post-transplantation relapse, we showed increased expression of the inhibitory molecules PD-L1, CD276/B7-H3, and CD155/PVRL2 in up to 40% of cases of relapse. PD-L1 overexpression on AML blasts impaired donor T cell functions *ex vivo*, and antileukemic responses could be partially restored upon treatment with anti-PD-L1 monoclonal antibody (41). It should be noted, however, that in most patients, the landscape of expression of inhibitory ligands at time of relapse was quite composite, with high inter-patient variability, hinting at the fact that blocking a single interaction might yield limited clinical benefits and that efforts should rather be aimed at identifying and targeting a shared regulator of these molecules. The relative frequency of changes in T cell costimulation molecules remained superimposable when analyzed in different cohorts of patients receiving allo-HCT from donors with variable levels of HLA-compatibility (37), similarly to patients experiencing downregulation of HLA class II molecules at relapse and differently from patients with genomic loss of HLA-haplotype.

However, to date, little is known about the molecular drivers of this phenotype in the post-transplantation setting, and most of the currently available knowledge relates to PD-L1 and its regulation in other malignancies. Activation of aberrant janus

kinase (JAK) signaling through 9p24.1 amplification has been shown to be a potent driver of PD-L1 upregulation in Hodgkin's lymphoma (56). Also, myeloproliferative neoplasms bearing the JAK^{V617F} point mutation had the same effect on PD-L1 expression (57). With that said, loss-of-function mutations in the JAK/STAT pathway observed in several other tumor types (e.g., melanoma) have been proven to be associated with resistance to PD-1/PD-L1 blockade (58–60). Also, Myc-driven lymphomas display constitutive upregulation of inhibitory molecules: Myc oncogenic signaling has been shown in fact to increase the expression of PD-L1 and of the "don't eat me" signal CD47 in tumor cells, impairing interactions with T lymphocytes and dendritic cells (61). Beside oncogenes driving PD-L1 overexpression, several epigenetic mechanisms have also been reported. Expression of PD-L1 has been shown, for instance, to be inversely correlated with methylation of its promoter and robustly induced upon treatment of tumor cells with hypomethylating agents (62). Also, micro RNAs (miRNAs), have been implicated in the regulation of PD-L1 expression by binding to the PD-L1 mRNA and driving its degradation; in AML, for instance, the levels of miRNA-34a showed inverse correlation with PD-L1 expression (63). Another emerging layer of regulation of PD-L1 is represented by post-translational modifications—for instance, through glycosylation of the mature protein (64).

In addition to all the tumor-intrinsic mechanisms of PD-L1 regulation mentioned in the previous paragraph, pro-inflammatory molecules (e.g., IFN-γ) secreted in the tumor microenvironment can also potently drive upregulation of PD-L1 on tumor cells (65). This might be extremely relevant in the setting of leukemia post-transplantation relapses, since, as discussed in the previous section, induction of a pro-inflammatory microenvironment conversely represents the key to reverting epigenetic downregulation of HLA class II molecules. Indeed, when data regarding expression of HLA molecules and inhibitory ligands at relapse in our patient cohorts were plotted together, it appeared quite evident that these two modalities of relapse are largely non-overlapping (41) and should prospectively be discriminated one from the other to enact the most appropriate salvage treatments.

Noticeably, the phenotypic features of T cells circulating in patients at the time of relapse mirror the changes observed in leukemic cells, with significant upregulation of inhibitory receptors in the patients whose leukemias express the respective ligands (41, 66). Recent studies showed that expression of inhibitory receptors such as PD-1 on T lymphocytes can at least in part be prompted by the intense stimulation conveyed to the donor immune system upon transfer into an allogeneic environment (67), as suggested also by the observation of higher expression of inhibitory receptors on the T cells of patients who received haplo-HCTs (66). However, in-depth analysis of T cells from patients who did or did not experience relapse allowed for the identification of specific exhaustion features in T cells from relapsing patients, with co-expression of multiple inhibitory receptors not only in terminally differentiated effectors, but also in early-differentiated memory stem and central memory T cells (66, 68). The exhausted phenotype was particularly evident in the patients' bone marrow, where T cell-leukemia interactions are mainly expected to occur and associated with a skewed T cell receptor (TCR) repertoire (66). Importantly, backtracking the clinical follow-up of patients who eventually relapsed, it was possible to identify the T cell exhaustion signature even months before relapse (41, 66) and in patients who relapsed after sole chemotherapy (69), suggesting that, upon further validation, these features might be used as an indicator to guide pre-emptive therapeutic approaches.

TUMOR-EXTRINSIC MECHANISMS OF RELAPSE

Beside altering their features to increase aggressiveness and reduce immunogenicity, malignant cells can also accelerate disease progression by rewiring the microenvironment to their advantage, coopting the niche and the physiological mechanisms at the basis of immune tolerance. Mostly investigated in the context of solid tumors, interactions between cancer cells and the microenvironment are also starting to gain more attention in hematological diseases and gain an additional layer of complexity upon allo-HCT, when the niche becomes an admixture of pathological and non-pathological elements of both host and donor origin (**Figure 2**).

One of the best characterized modalities employed by hematological tumors to alter the immune microenvironment that surrounds them is switching from the production of pro-inflammatory cytokines to the release of immunosuppressive molecules, including IL-10 and TGF-β. For instance, it has been shown that during transformation, myeloid cells can reduce their production of granulocyte colony-stimulating factor (G-CSF), IL-15, and IFN-γ. Defects in IFN-γ production have been correlated to a specific polymorphism, which has been also linked to clinical risk parameters (e.g., prednisone response) in patients affected by B-lineage acute lymphocytic leukemia (ALL) (70). Strongly produced by normal myeloid progenitors, the physiological function of IL-15 is to expand and activate effector T and NK cells (71) and to promote the generation of memory stem T cell subset (72). Therefore, it is not difficult to understand why high levels of this cytokine in the tumor microenvironment are unfavorable for leukemic cells. In the post-transplantation setting, low plasma levels of IL-15 have been correlated to higher risk of relapse in patient affected by different hematological malignancies (73). One recently discovered mechanism at the basis of the reduced production of IL-15 by AML cells is the internal tandem duplication (ITD) of the FLT3 tyrosine kinase (FLT3-ITD) (74).

Even though in non-transplantation setting, the dysregulated effect of several metabolites has been shown to mediate immune suppression. The expression of indoleamine 2,3-dioxygenase-1 (IDO1) by leukemia cells was for instance correlated with unfavorable prognosis in childhood AML (75). IDO1 is the first actor of an enzymatic cascade resulting in the inhibition of T cell function and the T regulatory cell reprogramming (76). Moreover, AML exhibits the ability to block T cell function through the amino acid arginase, which can also drive

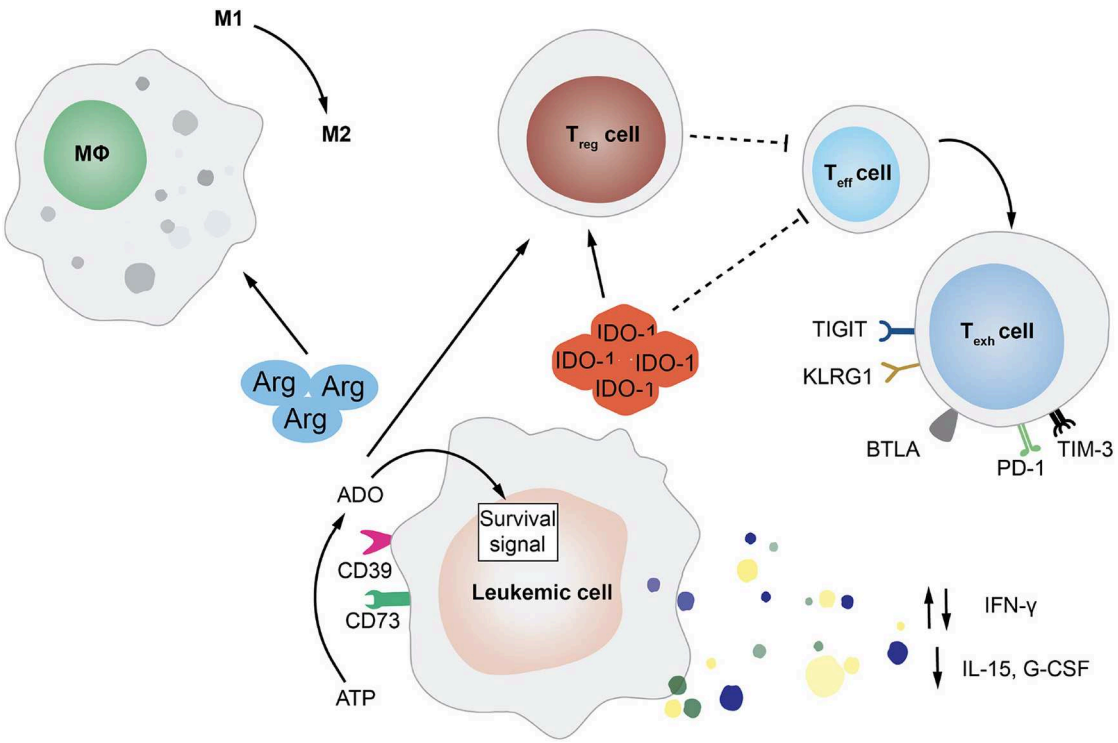

FIGURE 2 | Tumor-Extrinsic Mechanisms of Immune Evasion and Relapse. This cartoon summarizes several of the pathways exploited by leukemic cells in order to rewire the bone marrow microenvironment and evade immune recognition. In particular, featured in the figure are the deregulated release by AML blasts of cytokines, such as interferon-γ (IFN-γ), interleukin-15 (IL-15), and granulocyte-colony stimulating factor (G-CSF); the expression of enzymes involved in aminoacid metabolism, such as arginase (Arg) and indoleamine 2,3-dioxygenase (IDO-1); and the upregulation of the ectonucleotidases CD73 and CD39 that leads to the increase in extracellular adenosine (ADO). All of these mediators can have an impact on the frequency and function of immune cell subsets, impairing T and NK cell activity, driving effector T cells toward exhaustion, inducing the expansion of regulatory T cells (Treg), and promoting the phenotypic switch of macrophages from pro-inflammatory M1 to immuno-suppressive M2.

macrophages toward the suppressive M2-like phenotype (77). Other two enzymes that are gaining recent attention for their possible role in inducing leukemia immune escape are the ectonucleotidase CD73 (78) and the ectonucleoside triphosphate diphosphohydrolase-1 CD39 (79).

Recently, moreover, studies conducted in solid tumors highlighted a major role of tumor-induced metabolic remodeling in altering T cell state and function. Specifically, Vodnala et al. revealed that the elevated presence of extracellular potassium in the tumor microenvironment can promote a state of functional starvation in tumor-specific T cells. The starvation response results in induction of autophagy and in epigenetic reprogramming, impairing T cell differentiation and function (80).

TRANSLATING RELAPSE BIOLOGY INTO RATIONALES FOR TREATMENT

The ideal strategies to treat relapse after allo-HCT should exert both a direct anti-tumor activity and enhance the alloreactive GvL effect of allogeneic T cells, sparing the risks of inducing significant cytopenias, immunosuppression, or GvHD. Moreover, considering the numerous and complexly combined modalities of relapse that were summarized in previous section, it should be considered that, ideally only using combinatorial therapies, it might be possible to hit a target without exposing the flank to compensatory responses that ultimately select alternative mechanisms of escape.

Here, we summarize the most recent evidence about post-HCT relapse treatment modalities, categorizing strategies that rely on cellular therapies or that aim at boosting or redirecting the pre-existent donor-derived immune system.

Cellular Therapies

Donor Lymphocyte Infusions

One of the simplest and most intuitive ways to induce a GvL response after allogeneic HCT is to administer donor-lymphocyte infusions (DLIs). The main advantage of this strategy is the induction of a polyclonal T cell response able to target multiple antigens on malignant cells, reducing the risks of escaping T cell recognition just by loss of a single antigen. The major drawback of DLIs is represented by the possibility of donor T cells recognizing and attacking non-hematopoietic tissues, with the risk of triggering GvHD, which can turn out to be a serious, and often fatal, complication. This hazard can be significantly reduced either by incorporating suicide genes in the infused cells, acting as a "safety switch" in case of unwanted reactions (81, 82),

or by infusing specific T cell subsets endowed with lower intrinsic alloreactivity, such as memory (83, 84) or γδ (85, 86) T cells.

As discussed in previous sections, when considering the therapeutic use of DLI for relapses after haplo-HCT, it is fundamental to rapidly determine if relapse is sustained by HLA loss immune-escape leukemia variants that represent a clear counter indication to DLI administration. In fact, the genomic loss of the unshared HLA haplotype in leukemia cells not only renders them invisible to the major HLA alloreactivity exerted by infused T cells, but it also does not impact their recognition of healthy tissues, leaving the risk of DLI-induced GvHD largely unaltered. For these reasons, upon documentation of HLA loss, other salvage options should be prioritized as treatment strategies (17, 39).

Outside of this specific context, a considerable body of literature exists on the use of DLI as therapy of relapse after haplo-HCT. The first study after unmanipulated haplo-HCT performed under ATG-based GvHD prophylaxis utilized a median dose of donor T cells of 0.6×10^8 $CD3^+$/Kg, reporting significant risks of both severe acute GvHD (aGvHD, 30%) and chronic GvHD (cGvHD, 64%) (87). More recently, a study testing DLI after post-transplant cyclophosphamide (PTCy)-based GvHD prophylaxis yielded a 30% complete remission (CR) rate, with a risk of developing grade III–IV aGvHD or cGvHD of 15 and 8%, respectively. In this trial, a dose of 1×10^6 $CD3^+$/Kg was considered a reasonable starting dose (88). Another study showed that escalating doses of DLI after PTCy-based haplo-HCT were accompanied by at least a 33% CR rate, a 14% risk of grade II–III aGvHD, and no cases of grade III–IV aGvHD or cGvHD. In this study, the initial administered DLI dose was 1×10^5 $CD3^+$/Kg, in case of molecular relapse, and higher (from 1×10^6 rising to 1×10^7 $CD3^+$/Kg), in case of hematological relapse (89).

Several studies also reported results from combinatorial administration of DLI and immunomodulating agents, with the aim of increasing the immunogenicity of tumor cells, rendering them more susceptible to DLI action. The diverse dose schedules and time points of infusions preclude a clear guideline, but most trials employed a starting dose of 1×10^5 $CD3^+$/Kg, eventually escalating in the absence of GvHD development (88, 90–98).

Second Allogeneic Transplantation

Second allogeneic transplantation (allo-HCT2) to treat relapse after the first allo-HCT has recently gained more popularity, thanks to the introduction of reduced-intensity conditioning and improvements of supportive therapies, which have significantly reduced toxicities after allo-HCT2, historically burdened by treatment-related mortality up to 40–50% (99, 100). As in the case of DLI, when considering HCT2 for relapse after HLA-mismatched HCT, it is mandatory to discriminate whether relapse after the first transplant was classical or HLA loss. Especially in the second case, as mentioned above, selecting a second haploidentical donor with a different HLA haplotype provided some very promising preliminary results (40). Unfortunately, for all the studies on this subject, the inevitable selection bias of patients fit to receive a second conditioning and further transplantation must be taken into account, and clinical decisions must balance individual patient comorbidities and alternative therapeutic strategies.

Adoptive Immunotherapy With Genetically Redirected Immune Cells

Over the last few years, a number of landmark studies have demonstrated the feasibility and efficacy of using gene therapy to redirect immune cells in a non-HLA-restricted fashion against antigens of choice. The most striking example is provided by chimeric antigen receptor (CAR) T cells, which are capable of binding to the surface antigen of choice without the need for TCR-HLA interactions, thus representing a promising therapeutic option for patients relapsing with HLA loss or HLA downregulation. Moreover, CAR potent synthetic co-stimulatory domains may bypass the effect of the immune-suppressive signals expressed by tumor cells or microenvironment (101, 102).

CAR T cells targeting CD19 are, to date, the best studied and have demonstrated significant activity in chemotherapy refractory CLL, B-cell lymphomas and B-ALL in the autologous setting (103–108). There is also growing evidence of the efficacy of donor-origin CD19 CAR T cells in patients relapsing after allo-HCT (102, 108–111) or even haplo-HCT (112, 113). In this scenario, the infusion of allogeneic CAR T cells could carry the theoretical risk of GvHD; however, incidence of this fearsome complication in early trials was quite low, and an elegant study in mouse models showed that the CAR-driven and TCR-driven signal actually adds up, accelerating exhaustion and limiting alloreactions (101). Still, a number of studies are focusing on the development of improved strategies to further enhance CAR T efficacy and persistence without risking to induce GvHD, such as by transducing recipient-derived donor T cells (113), by using genome editing approaches to knock out the endogenous TCR (101, 114), or by modifying with the CAR different immune cells, less prone to induce GvHD (85, 115, 116).

Redirecting or Boosting the Donor-Derived Immune System

Bispecific Antibodies

Based on a principle similar to the one that guided the development of CAR T cells, bispecific antibodies can also enable redirection of immune cells toward malignant cells, forcing the formation of an immunological synapsis through the binding of an antigen expressed on effectors (such as CD3 on T cells or CD16 on NK cells), with one expressed by the tumor target (such as CD19 for lymphoid malignancies or CD33 for myeloid leukemias) (117, 118). This results in the release of cytotoxic granules in close proximity to target cells, with the ultimate step of apoptosis induction and elimination, also fueled by inflammatory cytokines production and antigen spreading mechanisms (119). This strategy could be another useful way to circumvent HLA-restriction of TCR, with the potential added value of being readily available off the shelf and taking advantage of cells that are already circulating in the patient and tolerized against his healthy tissues. However, other immune-evasion mechanisms (related to the induction of inhibitory checkpoint molecules and the production of immunosuppressive cytokines

or metabolites), have been shown to rapidly emerge upon treatment with bispecifics, suggesting that full exploitation of the anti-tumoral activity of these promising molecules could pass by enhancing costimulatory pathways or blocking immune checkpoints (120–125).

Epigenetic Therapies

The two commercially available hypomethylating agents (HMAs), azacytidine (Aza) and decitabine (DAC), are frequently used for post-HCT relapse treatment in AML or myelodysplastic syndromes (MDS). HMAs indirectly inhibit DNA methyltransferases significantly altering DNA methylation patterns with consequent induction of cell cycle arrest, DNA damage accumulation, apoptosis, and differentiation (126–132). More recently, immune-related effects of hypomethylating agents have also been described. In particular, Aza stimulates antitumor immunity inducing the upregulation on leukemic cells of leukemia-associated and minor-histocompatibility antigens, including PRAME, MAGE-A, NY-ESO1, and HA-1 (129, 130, 133–136). Aza can also lead to increased HLA class-I and II expression and modulate tumor-immunogenicity through the upregulation on leukemic cell surface of costimulatory molecules, such as CD80, CD86, ULBP, and MIC-A (137). Among the reported effects, HMAs can induce the expression of important players involved in anti-viral responses, including IFN-γ and cytokines. Interestingly, Aza can also promote upregulation of endogenous retroviral elements on tumor cells, inducing a "viral mimicry" response that ultimately results in the induction of anti-tumor immunity (138–140). However, Aza can also act as a double-edged sword, since it can upregulate PD-1, PD-L1/L2, and CTLA-4 inhibitory pathways and induce the expansion of regulatory T cells (131), potentially hampering the intensity and duration of cytotoxic T cell responses and facilitating the tolerization and exhaustion of tumor-specific T cells (141, 142).

Due to their reported immune-related effects, HMAs have been frequently employed in combination with DLI. To date, we have data on more than 600 patients undergoing salvage regimens including Aza and DLI, reporting very variable results in terms of clinical outcome (143–148). Because of the heterogeneous results obtained so far and lack of consent on treatment schedules, two retrospective surveys have analyzed the correlates of efficacy of Aza+DLI combinations in more homogeneous cohorts, one facilitated by the German Cooperative Transplant Study Group (145) and the other by the EBMT (146). These studies reported that patients that benefitted the most from Aza+DLI combinatorial approach were those who presented low disease burden at the time of relapse (molecular relapse or <20% blasts in bone marrow) and those with a longer interval from allo-HCT to relapse. These variables can be adopted to predict treatment response through a score assignment (AZA relapse prognostic score: ARPS), even if an independent validation cohort is still lacking (146).

Histone acetylation is another epigenetic mechanism of immune regulation, balanced between the activity of histone acetyl-transferases (HATs) and histone deacetylases (HDACs). HDAC inhibitors, such as vorinostat and panobinostat, have been associated with the upregulation of major-histocompatibility and co-stimulatory molecules on AML cell surface through the induction of an open and readable structure of chromatin (149, 150). To date, two prospective phase I/II trials of post-HCT therapy with panobinostat for AML/MDS patients, alone or in combination with DAC and DLI, have been reported (151, 152).

Immune Checkpoint Blockade

Immune checkpoint inhibition through the administration of monoclonal antibodies that target the PD-1/PD-L1 and CTLA-4/B7 axes is emerging as an attractive strategy to enhance alloreactive T cell function and rewire the immunosuppressive milieu in which disease relapse often occurs (153–155). Clinical trials exploring the efficacy of immune checkpoint inhibitors after allo-HCT have shown some promise using the anti-CTLA4 antibody ipilimumab (156, 157) and more modest results using PD-1 inhibitors in diseases other than Hodgkin's lymphoma (158–161). Moreover, post-transplantation treatment with checkpoint inhibitors appears to be associated to a significant risk of severe and treatment-refractory GvHD and immune-related events (162).

However, as the balance of stimulatory and inhibitory signals determines the magnitude of immune responses against tumor cells, combining HMAs and immune-checkpoint blockade therapies may represent an interesting approach to release the "break" signal received by tumor-reactive immune cells (163–165). A phase II trial exploring the combination of the anti-PD1 monoclonal antibody Nivolumab and Aza in relapsed AML reported an overall response rate of 33% (166), and several ongoing trials are assessing the efficacy of HMAs and immune checkpoint inhibitor combinations, some of them recruiting also post-transplantation relapsed patients (NCT02890329, NCT02845297, NCT02996474, and NCT02397720).

Cytokine Therapies

The use of exogenous cytokines to boost or restore T cell- and NK cell-impaired effector functions have been object of intense investigation in cancer therapy and especially in the field of hematological malignancies. Interleukin 2 (IL2), IFN-α, and IL-15 are the best studied. IL-2 has been shown to stimulate the anti-tumor effect of lymphocytes, polarizing helper T cell responses toward type 1 and exerting both immune-enhancing and immune-suppressive activities (167–169). However, application of IL-2 monotherapy against AML has yielded very limited clinical benefit, both for the induction of regulatory T cells that impaired antileukemic activity and for the rapid drop in effector functions due to T cells terminal differentiation and exhaustion (170–173). IFN-α, however, exerts pleiotropic functions, since it has a direct antileukemic effect and also possesses immune-stimulatory properties, leading to dendritic cells stimulation, enhancement of NK-cell cytotoxicity, and sensitization of T cells to other inflammatory cytokines, such as IL-2 (174–177). Despite these theoretical premises, IFN-α failed to demonstrate significant activity as single agent in post-transplantation relapse (178–181). As previously described, IL-15 is a potent immunostimulatory cytokine, that potentiates both T and NK cell immune responses, promoting the generation and

maintenance of high-avidity and long-lived $CD8^+$ memory T cells. IL-15 also prevents activation-induced T cell death and does not induce the expansion of immunosuppressive regulatory T cells (72, 182–186). A phase I trial testing the IL-15 super-agonist complex ALT-803 in patients relapsing after allo-HCT showed a very promising response rate (19% of evaluable patients), correlated to the expansion of both NK and T cells (187). Recently, novel approaches to transfer high concentration of cytokines to the tumor site and reduce their systemic effects are emerging, including gene therapy "Trojan Horse" strategies (188) and the use of lipid nanoparticles to convey to the tumor site mRNAs encoding cytokines (189).

Immune-Related Effects of Targeted Therapies

The growing armamentarium of targeted therapies is providing new evidence that, beside their direct effects, some of them can also promote antitumor immunity. A recent work testing the effect of the tyrosine-kinase inhibitor sorafenib in a mouse model of leukemia showed that the treatment increased the production of IL-15 by leukemic cells bearing FLT3-ITD. This resulted in enhanced $CD8^+$ T cell effector function (via their increased metabolic capacity) and leukemia eradication. Mechanistically, sorafenib induced transcription of IL-15, acting by inhibition of the transcription factor ATF435 that in turn suppresses the IL-15 activator interferon regulatory factor 7 (IRF7) (74).

Another example of tyrosine-kinase inhibitor exerting "off-target" immune mechanisms is represented by imatinib, which is indicated in Philadelphia-positive (Ph+) leukemias, namely chronic myeloid leukemia (CML) and Ph+ acute lymphoblastic leukemia (ALL). Allogeneic HCT remains the only curative option for Ph+ ALL and advanced-phase CML, and there is general consensus about imatinib administration following HCT (190, 191). In addition to targeting Bcr/Abl1 and KIT oncogene products, imatinib modulates the proliferation, polarization, and functionality of different subsets of myeloid and lymphoid cells (192–195). This modulation can exert either inhibitory or stimulating immune effects. Among the inhibitory effects are the inhibition of dendritic cells expansion, resulting in less efficient priming of cytotoxic T cells (196–199), the polarization toward a M2-like anti-inflammatory phenotype of tumor-associated macrophages (200, 201), the reduction of effector-cytokine production by $CD4^+$ T cells in response to TCR-signaling (202, 203), and the reduction of IgM-producing memory B-cell frequency (204–206). On the other hand, imatinib also has stimulating effects such as decreased expression of 2,3-IDO and consequent apoptosis in regulatory T cells (207, 208); reduction of myeloid-derived suppressor cells, thus restoring a T cell cytotoxic response (209–211); reduced secretion of VEGF with subsequent antiangiogenic effect (212, 213); polarization toward a higher Th1/Th2 ratio (214–216); and preferential expression of activating NK receptors (217).

CONCLUSIONS AND PERSPECTIVES

The landscape of allo-HCT, and haplo-HCT in particular, is rapidly changing, with multiple platforms able to achieve remarkable long-term outcome results. The reduced risk of treatment-related toxicities and mortality has also opened the possibility to implement innovative pharmacological or cellular therapies in the post-transplantation follow-up, transforming the perception of allo-HCT from that of a final consolidation therapy to a "platform" to build on. In this new scenario, it will be of utmost relevance to also associate to the analysis of clinical endpoints a detailed study on how changing the recipe of allo-HCT influences its immunobiology. For instance, understanding the relative contribution of each immune cell subset transferred as part of the graft in the induction of GvHD and protection against relapse will be fundamental to guide further improvements in "tailoring" graft composition and post-transplantation cell therapies, as convincingly suggested by a number of recent studies (218–220). It is now evident that the success or failure of transplantation is linked to our ability to take full advantage of the many features endowed in the immune system and to combine them with targeted therapies to hit as many tumor targets as possible, reducing the chances of selection of escape variants. Generation of new quantitative systems to map tumor immune targets, characterization of the tumor immune microenvironment by multi-omics single-cell technologies, and generation of more refined humanized mouse model to mirror allo-HCT all appear to be promising avenues in advancing knowledge on allo-HCT immunobiology and, ultimately, in generating new rationales to further improve clinical outcome.

AUTHOR CONTRIBUTIONS

PR, VG, and FL reviewed available literature and drafted the paper. FC and LV provided critical discussion and revised the manuscript draft.

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REFERENCES

1. Kanakry CG, Fuchs EJ, Luznik L. Modern approaches to HLA-haploidentical blood or marrow transplantation. *Nat Rev Clin Oncol*. (2016) 13:10–24. doi: 10.1038/nrclinonc.2015.128
2. Patriarca F, Luznik L, Medeot M, Zecca M, Bacigalupo A, Di Bartolomeo P, et al. Experts' considerations on HLA-haploidentical stem cell transplantation. *Eur J Haematol*. (2014) 93:187–97. doi: 10.1111/ejh.12322
3. Aversa F, Pierini A, Ruggeri L, Martelli MF, Velardi A. The evolution of T cell depleted haploidentical transplantation. *Front Immunol*. (2019) 10:2769. doi: 10.3389/fimmu.2019.02769
4. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood*. (2014) 124:638–44. doi: 10.1182/blood-2014-03-564401
5. Mancusi A, Piccinelli S, Velardi A, Pierini A. CD4+FOXP3+ Regulatory T cell therapies in HLA haploidentical hematopoietic transplantation. *Front Immunol*. (2019) 10:2901. doi: 10.3389/fimmu.2019.02901
6. Ciceri F, Bonini C, Stanghellini MTL, Bondanza A, Traversari C, Salomoni M, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I–II study. *Lancet Oncol*. (2009) 10:489–500. doi: 10.1016/S1470-2045(09)70074-9
7. Lucarelli B, Bertaina A, Merli P, Quintarelli C, Pende D, Falco M, et al. T-cell depleted HLA-haploidentical allogeneic hematopoietic stem cell transplantation (haplo-HSCT) followed by donor lymphocyte infusion with T cells transduced with the inducible caspase 9 (iC9) suicide gene in children with hematological malignancies. *Blood*. (2016) 128:4683. doi: 10.1182/blood.V128.22.4683.4683
8. Zhang P, Tey S-K. Adoptive T cell therapy following haploidentical hematopoietic stem cell transplantation. *Front Immunol*. (2019) 10:1854. doi: 10.3389/fimmu.2019.01854
9. Huang X-J, Liu D-H, Liu K-Y, Xu L-P, Chen H, Han W, et al. Haploidentical hematopoietic stem cell transplantation without *in vitro* T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant*. (2006) 38:291–7. doi: 10.1038/sj.bmt.1705445
10. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, G et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
11. Passweg JR, Baldomero H, Basak GW, Chabannon C, Corbacioglu S, Duarte R, et al. The EBMT activity survey report 2017: a focus on allogeneic HCT for nonmalignant indications and on the use of non-HCT cell therapies. *Bone Marrow Transplant*. (2019) 54:1575–85. doi: 10.1038/s41409-019-0465-9
12. D'Souza A, Fretham C. *Current Uses and Outcomes of Hematopoietic Cell Transplantation (HCT): CIBMTR Summary Slides, 2019*. Available online at: https://www.cibmtr.org (accessed February 19, 2020)
13. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-vs.-leukemia reactions after bone marrow transplantation. *Blood*. (1990) 75:555–62. doi: 10.1182/blood.V75.3.555.555
14. Garrido F, Perea F, Bernal M, Sánchez-Palencia A, Aptsiauri N, Ruiz-Cabello F. The escape of cancer from T cell-mediated immune surveillance: HLA class I loss and tumor tissue architecture. *Vaccines*. (2017) 5:7. doi: 10.3390/vaccines5010007
15. Masuda K, Hiraki A, Fujii N, Watanabe T, Tanaka M, Matsue K, et al. Loss or down-regulation of HLA class I expression at the allelic level in freshly isolated leukemic blasts. *Cancer Sci*. (2007) 98:102–8. doi: 10.1111/j.1349-7006.2006.00356.x
16. Brouwer RE, van der Heiden P, Schreuder GMT, Mulder A, Datema G, Anholts JDH, et al. Loss or downregulation of HLA class I expression at the allelic level in acute leukemia is infrequent but functionally relevant, and can be restored by interferon. *Hum Immunol*. (2002) 63:200–10. doi: 10.1016/S0198-8859(01)00381-0
17. Vago L, Perna SK, Zanussi M, Mazzi B, Barlassina C, Stanghellini MTL, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med*. (2009) 361:478–88. doi: 10.1056/NEJMoa0811036
18. Mazzi B, Clerici TD, Zanussi M, Lupo Stanghellini MT, Vago L, Sironi E, et al. Genomic typing for patient-specific human leukocyte antigen-alleles is an efficient tool for relapse detection of high-risk hematopoietic malignancies after stem cell transplantation from alternative donors. *Leukemia*. (2008) 22:2119–22. doi: 10.1038/leu.2008.98
19. Makishima H, Maciejewski JP. Pathogenesis and consequences of uniparental disomy in cancer. *Clin Cancer Res*. (2011) 17:3913–23. doi: 10.1158/1078-0432.CCR-10-2900
20. O'Keefe C, McDevitt MA, Maciejewski JP. Copy neutral loss of heterozygosity: a novel chromosomal lesion in myeloid malignancies. *Blood*. (2010) 115:2731–9. doi: 10.1182/blood-2009-10-201848
21. Tuna M, Knuutila S, Mills GB. Uniparental disomy in cancer. *Trends Mol Med*. (2009) 15:120–8. doi: 10.1016/j.molmed.2009.01.005
22. Villalobos IB, Takahashi Y, Akatsuka Y, Muramatsu H, Nishio N, Hama A, et al. Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation. *Blood*. (2010) 115:3158–61. doi: 10.1182/blood-2009-11-254284
23. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. (1986) 319:675–8. doi: 10.1038/319675a0
24. Barrett J, Blazar BR. Genetic trickery — escape of leukemia from immune attack. *N Engl J Med*. (2009) 361:524–5. doi: 10.1056/NEJMe0903177
25. Gupta M, Raghavan M, Gale RE, Chelala C, Allen C, Molloy G, et al. Novel regions of acquired uniparental disomy discovered in acute myeloid leukemia. *Genes Chromoso Cancer*. (2008) 47:729–39. doi: 10.1002/gcc.20573
26. Dubois V, Sloan-Béna F, Cesbron A, Hepkema BG, Gagne K, Gimelli S, et al. Pretransplant HLA mistyping in diagnostic samples of acute myeloid leukemia patients due to acquired uniparental disomy. *Leukemia*. (2012) 26:2079–85. doi: 10.1038/leu.2012.68
27. Pereira S, Vayntrub T, Hiraki DD, Cherry AM, Arai S, Dvorak CC, et al. Short tandem repeat and human leukocyte antigen mutations or losses confound engraftment and typing analysis in hematopoietic stem cell transplants. *Hum Immunol*. (2011) 72:503–9. doi: 10.1016/j.humimm.2011.03.003
28. Crucitti L, Crocchiolo R, Toffalori C, Mazzi B, Greco R, Signori A, et al. Incidence, risk factors and clinical outcome of leukemia relapses with loss of the mismatched HLA after partially incompatible hematopoietic stem cell transplantation. *Leukemia*. (2015) 29:1143–52. doi: 10.1038/leu.2014.314
29. McCurdy SR, Iglehart BS, Batista DA, Gocke CD, Ning Y, Knaus HA, et al. Loss of the mismatched human leukocyte antigen haplotype in two acute myelogenous leukemia relapses after haploidentical bone marrow transplantation with post-transplantation cyclophosphamide. *Leukemia*. (2016) 30:2102–6. doi: 10.1038/leu.2016.144
30. Grosso D, Johnson E, Colombe B, Alpdogan O, Carabasi M, Filicko-O'Hara J, et al. Acquired uniparental disomy in chromosome 6p as a feature of relapse after T-cell replete haploidentical hematopoietic stem cell transplantation using cyclophosphamide tolerization. *Bone Marrow Transplant*. (2017) 52:615–9. doi: 10.1038/bmt.2016.324
31. Peccatori J, Forcina A, Clerici D, Crocchiolo R, Vago L, Stanghellini MTL, et al. Sirolimus-based graft-vs.-host disease prophylaxis promotes the *in vivo* expansion of regulatory T cells and permits peripheral blood stem cell transplantation from haploidentical donors. *Leukemia*. (2015) 29:396–405. doi: 10.1038/leu.2014.180
32. Cieri N, Greco R, Crucitti L, Morelli M, Giglio F, Levati G, et al. Post-transplantation cyclophosphamide and sirolimus after haploidentical hematopoietic stem cell transplantation using a treosulfan-based myeloablative conditioning and peripheral blood stem cells. *Biol Blood Marrow Transplant*. (2015) 21:1506–14. doi: 10.1016/j.bbmt.2015.04.025
33. Toffalori C, Cavattoni I, Deola S, Mastaglio S, Giglio F, Mazzi B, et al. Genomic loss of patient-specific HLA in acute myeloid leukemia relapse after well-matched unrelated donor HSCT. *Blood*. (2012) 119:4813–5. doi: 10.1182/blood-2012-02-411686
34. Waterhouse M, Pfeifer D, Pantic M, Emmerich F, Bertz H, Finke J. Genome-wide profiling in AML patients relapsing after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. (2011) 17:1450–9.e1. doi: 10.1016/j.bbmt.2011.07.012

35. Hamdi A, Cao K, Poon LM, Aung F, Kornblau S, Fernandez Vina MA, et al. Are changes in HLA Ags responsible for leukemia relapse after HLA-matched allogeneic hematopoietic SCT? *Bone Marrow Transplant*. (2015) 50:411–3. doi: 10.1038/bmt.2014.285
36. Jan M, Leventhal MJ, Morgan EA, Wengrod JC, Nag A, Drinan SD, et al. Recurrent genetic HLA loss in AML relapsed after matched unrelated allogeneic hematopoietic cell transplantation. *Blood Adv*. (2019) 3:2199–204. doi: 10.1182/bloodadvances.2019000445
37. Tsirigotis P, Byrne M, Schmid C, Baron F, Ciceri F, Esteve J, et al. Relapse of AML after hematopoietic stem cell transplantation: methods of monitoring and preventive strategies. a review from the ALWP of the EBMT. *Bone Marrow Transplant*. (2016) 51:1431–38. doi: 10.1038/bmt.2016.167
38. Ahci M, Toffalori C, Bouwmans E, Crivello P, Brambati C, Pultrone C, et al. A new tool for rapid and reliable diagnosis of HLA loss relapses after HSCT. *Blood*. (2017) 130:1270–3. doi: 10.1182/blood-2017-05-784306
39. Vago L, Ciceri F. Choosing the alternative. *Biol Blood Marrow Transplant*. (2017) 23:1813–4. doi: 10.1016/j.bbmt.2017.09.009
40. Imus PH, Blackford AL, Bettinotti M, Iglehart B, Dietrich A, Tucker N, et al. Major histocompatibility mismatch and donor choice for second allogeneic bone marrow transplantation. *Biol Blood Marrow Transplant*. (2017) 23:1887–94. doi: 10.1016/j.bbmt.2017.07.014
41. Toffalori C, Zito L, Gambacorta V, Riba M, Oliveira G, Bucci G, et al. Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nat Med*. (2019) 25:603–11. doi: 10.1038/s41591-019-0400-z
42. Christopher MJ, Petti AA, Rettig MP, Miller CA, Chendamarai E, Duncavage EJ, et al. Immune escape of relapsed AML cells after allogeneic transplantation. *N Engl J Med*. (2018) 379:2330–41. doi: 10.1056/NEJMoa1808777
43. Stevanović S, van Schie MLJ, Griffioen M, Falkenburg JH. HLA-class II disparity is necessary for effective T cell mediated graft-vs.-leukemia effects in NOD/scid mice engrafted with human acute lymphoblastic leukemia. *Leukemia*. (2013) 27:985–7. doi: 10.1038/leu.2012.270
44. Ennishi D, Takata K, Béguelin W, Duns G, Mottok A, Farinha P, et al. Molecular and genetic characterization of MHC deficiency identifies EZH2 as therapeutic target for enhancing immune recognition. *Cancer Discov*. (2019) 9:546–63. doi: 10.1158/2159-8290.CD-18-1090
45. Rimsza LM, Roberts RA, Miller TP, Unger JM, LeBlanc M, Braziel RM, et al. Loss of MHC class II gene and protein expression in diffuse large B-cell lymphoma is related to decreased tumor immunosurveillance and poor patient survival regardless of other prognostic factors: a follow-up study from the leukemia and lymphoma molecular profiling project. *Blood*. (2004) 103:4251–8. doi: 10.1182/blood-2003-07-2365
46. Løvig T, Andersen SN, Thorstensen L, Diep CB, Meling GI, Lothe RA, et al. Strong HLA-DR expression in microsatellite stable carcinomas of the large bowel is associated with good prognosis. *Br J Cancer*. (2002) 87:756–62. doi: 10.1038/sj.bjc.6600507
47. Roemer MGM, Redd RA, Cader FZ, Pak CJ, Abdelrahman S, Ouyang J, et al. Major histocompatibility complex class II and programmed death ligand 1 Expression predict outcome after programmed death 1 blockade in classic hodgkin lymphoma. *J Clin Oncol*. (2018) 36:942–50. doi: 10.1200/JCO.2017.77.3994
48. Lansford JL, Dharmasiri U, Chai S, Hunsucker SA, Bortone DS, Keating JE, et al. Computational modeling and confirmation of leukemia-associated minor histocompatibility antigens. *Blood Adv*. (2018) 2:2052–62. doi: 10.1182/bloodadvances.2018022475
49. Salman A, Koparde V, Hall CE, Jameson-Lee M, Roberts C, Serrano M, et al. Determining the quantitative principles of T cell response to antigenic disparity in stem cell transplantation. *Front Immunol*. (2018) 9:2284. doi: 10.3389/fimmu.2018.02284
50. Steidl C, Shah SP, Woolcock BW, Rui L, Kawahara M, Farinha P, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. (2011) 471:377–81. doi: 10.1038/nature09754
51. Cycon KA, Mulvaney K, Rimsza LM, Persky D, Murphy SP. Histone deacetylase inhibitors activate CIITA and MHC class II antigen expression in diffuse large B-cell lymphoma. *Immunology*. (2013) 140:259–72. doi: 10.1111/imm.12136
52. Tarafdar A, Hopcroft LEM, Gallipoli P, Pellicano F, Cassels J, Hair A, et al. CML cells actively evade host immune surveillance through cytokine-mediated downregulation of MHC-II expression. *Blood*. (2017) 129:199–208. doi: 10.1182/blood-2016-09-742049
53. Naka K, Hoshii T, Muraguchi T, Tadokoro Y, Ooshio T, Kondo Y, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature*. (2010) 463:676–80. doi: 10.1038/nature08734
54. Lee YJ, Han Y, Lu HT, Nguyen V, Qin H, Howe PH, et al. TGF-beta suppresses IFN-gamma induction of class II MHC gene expression by inhibiting class II transactivator messenger RNA expression. *J Immunol*. (1997) 158:2065–75.
55. Norde WJ, Maas F, Hobo W, Korman A, Quigley M, Kester MGD, et al. PD-1/PD-L1 interactions contribute to functional T-cell impairment in patients who relapse with cancer after allogeneic stem cell transplantation. *Cancer Res*. (2011) 71:5111–22. doi: 10.1158/0008-5472.CAN-11-0108
56. Green MR, Monti S, Rodig SJ, Juszczynski P, Currie T, O'Donnell E, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood*. (2010) 116:3268–77. doi: 10.1182/blood-2010-05-282780
57. Prestipino A, Emhardt AJ, Aumann K, O'Sullivan D, Gorantla SP, Duquesne S, et al. Oncogenic JAK2V617F causes PD-L1 expression, mediating immune escape in myeloproliferative neoplasms. *Sci Transl Med*. (2018) 10:eaam7729. doi: 10.1126/scitranslmed.aam7729
58. Shin DS, Zaretsky JM, Escuin-Ordinas H, Garcia-Diaz A, Hu-Lieskovan S, Kalbasi A, et al. Primary resistance to PD-1 blockade mediated by JAK1/2 mutations. *Cancer Discov*. (2017) 7:188–201. doi: 10.1158/2159-8290.CD-16-1223
59. Zaretsky JM, Garcia-Diaz A, Shin DS, Escuin-Ordinas H, Hugo W, Hu-Lieskovan S, et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. *N Engl J Med*. (2016) 375:819–29. doi: 10.1056/NEJMoa1604958
60. Sucker A, Zhao F, Pieper N, Heeke C, Maltaner R, Stadtler N, et al. Acquired IFNγ resistance impairs anti-tumor immunity and gives rise to T-cell-resistant melanoma lesions. *Nat Commun*. (2017) 8:15440. doi: 10.1038/ncomms15440
61. Casey SC, Tong L, Li Y, Do R, Walz S, Fitzgerald KN, et al. MYC regulates the antitumor immune response through CD47 and PD-L1. *Science*. (2016) 352:227–31. doi: 10.1126/science.aac9935
62. Wrangle J, Wang W, Koch A, Easwaran H, Mohammad HP, Vendetti F, et al. Alterations of immune response of non-small cell lung cancer with azacytidine. *Oncotarget*. (2013) 4:2067–79. doi: 10.18632/oncotarget.1542
63. Wang X, Li J, Dong K, Lin F, Long M, Ouyang Y, et al. Tumor suppressor miR-34a targets PD-L1 and functions as a potential immunotherapeutic target in acute myeloid leukemia. *Cell Signal*. (2015) 27:443–52. doi: 10.1016/j.cellsig.2014.12.003
64. Li C-W, Lim S-O, Xia W, Lee H-H, Chan L-C, Kuo C-W, et al. Glycosylation and stabilization of programmed death ligand-1 suppresses T-cell activity. *Nat Commun*. (2016) 7:12632. doi: 10.1038/ncomms12632
65. Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood*. (2007) 110:296–304. doi: 10.1182/blood-2006-10-051482
66. Noviello M, Manfredi F, Ruggiero E, Perini T, Oliveira G, Cortesi F, et al. Bone marrow central memory and memory stem T-cell exhaustion in AML patients relapsing after HSCT. *Nat Commun*. (2019) 10:1065. doi: 10.1038/s41467-019-08871-1
67. Simonetta F, Pradier A, Bosshard C, Masouridi-Levrat S, Dantin C, Koutsi A, et al. Dynamics of expression of programmed cell death protein-1 (PD-1) on T cells after allogeneic hematopoietic stem cell transplantation. *Front Immunol*. (2019) 10:1034. doi: 10.3389/fimmu.2019.01034
68. Hutten TJA, Norde WJ, Woestenenk R, Wang RC, Maas F, Kester M, et al. Increased coexpression of PD-1, TIGIT, and KLRG-1 on tumor-reactive CD8+ T Cells during relapse after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant*. (2018) 24:666–77. doi: 10.1016/j.bbmt.2017.11.027
69. Knaus HA, Berglund S, Hackl H, Blackford AL, Zeidner JF, Montiel-Esparza R, et al. Signatures of CD8+ T cell dysfunction in AML patients and their

reversibility with response to chemotherapy. *JCI Insight*. (2018) 3:120974. doi: 10.1172/jci.insight.120974
70. Cloppenborg T, Stanulla M, Zimmermann M, Schrappe M, Welte K, Klein C. Immunosurveillance of childhood ALL: polymorphic interferon-gamma alleles are associated with age at diagnosis and clinical risk groups. *Leukemia*. (2005) 19:44–8. doi: 10.1038/sj.leu.2403553
71. Colpitts SL, Stonier SW, Stoklasek TA, Root SH, Aguila HL, Schluns KS, et al. Transcriptional regulation of IL-15 expression during hematopoiesis. *J Immunol*. (2013) 191:3017–24. doi: 10.4049/jimmunol.1301389
72. Cieri N, Camisa B, Cocchiarella F, Forcato M, Oliveira G, Provasi E, et al. IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood*. (2013) 121:573–84. doi: 10.1182/blood-2012-05-431718
73. Thiant S, Yakoub-Agha I, Magro L, Trauet J, Coiteux V, Jouet J-P, et al. Plasma levels of IL-7 and IL-15 in the first month after myeloablative BMT are predictive biomarkers of both acute GVHD and relapse. *Bone Marrow Transplant*. (2010) 45:1546–52. doi: 10.1038/bmt.2010.13
74. Mathew NR, Baumgartner F, Braun L, O'Sullivan D, Thomas S, Waterhouse M, et al. Sorafenib promotes graft-vs.-leukemia activity in mice and humans through IL-15 production in FLT3-ITD-mutant leukemia cells. *Nat Med*. (2018) 24:282–91. doi: 10.1038/nm.4484
75. Folgiero V, Goffredo BM, Filippini P, Masetti R, Bonanno G, Caruso R, et al. Indoleamine 2,3-dioxygenase 1 (IDO1) activity in leukemia blasts correlates with poor outcome in childhood acute myeloid leukemia. *Oncotarget*. (2014) 5:2052–64. doi: 10.18632/oncotarget.1504
76. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity*. (2005) 22:633–42. doi: 10.1016/j.immuni.2005.03.013
77. Mussai F, De Santo C, Abu-Dayyeh I, Booth S, Quek L, McEwen-Smith RM, et al. Acute myeloid leukemia creates an arginase-dependent immunosuppressive microenvironment. *Blood*. (2013) 122:749–58. doi: 10.1182/blood-2013-01-480129
78. Serra S, Horenstein AL, Vaisitti T, Brusa D, Rossi D, Laurenti L, et al. CD73-generated extracellular adenosine in chronic lymphocytic leukemia creates local conditions counteracting drug-induced cell death. *Blood*. (2011) 118:6141–52. doi: 10.1182/blood-2011-08-374728
79. Dulphy N, Henry G, Hemon P, Khaznadar Z, Dombret H, Boissel N, et al. Contribution of CD39 to the immunosuppressive microenvironment of acute myeloid leukaemia at diagnosis. *Br J Haematol*. (2014) 165:722–5. doi: 10.1111/bjh.12774
80. Vodnala SK, Eil R, Kishton RJ, Sukumar M, Yamamoto TN, Ha N-H, et al. T cell stemness and dysfunction in tumors are triggered by a common mechanism. *Science*. (2019) 363:eaau0135. doi: 10.1126/science.aau0135
81. Zhou X, Brenner MK. Improving the safety of T-Cell therapies using an inducible caspase-9 gene. *Exp Hematol*. (2016) 44:1013–9. doi: 10.1016/j.exphem.2016.07.011
82. Greco R, Oliveira G, Stanghellini MTL, Vago L, Bondanza A, Peccatori J, et al. Improving the safety of cell therapy with the TK-suicide gene. *Front Pharmacol*. (2015) 6:95. doi: 10.3389/fphar.2015.00095
83. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, et al. Memory CD4+ T cells do not induce graft-vs.-host disease. *J Clin Investig*. (2003) 112:101–8. doi: 10.1172/JCI17601
84. Muffly L, Sheehan K, Armstrong R, Jensen K, Tate K, Rezvani AR, et al. Infusion of donor-derived CD8+ memory T cells for relapse following allogeneic hematopoietic cell transplantation. *Blood Adv*. (2018) 2:681–90. doi: 10.1182/bloodadvances.2017012104
85. Sebestyen Z, Prinz I, Déchanet-Merville J, Silva-Santos B, Kuball J. Translating gammadelta (γδ) T cells and their receptors into cancer cell therapies. *Nat Rev Drug Discov*. (2019) 24:666–77. doi: 10.1038/s41573-019-0038-z
86. Locatelli F, Merli P, Rutella S. At the bedside: innate immunity as an immunotherapy tool for hematological malignancies. *J Leukocyte Biol*. (2013) 94:1141–57. doi: 10.1189/jlb.0613343
87. Huang X-J, Liu D-H, Liu K-Y, Xu L-P, Chen H, Han W. Donor lymphocyte infusion for the treatment of leukemia relapse after HLA-mismatched/haploidentical T-cell-replete hematopoietic stem cell transplantation. *Haematologica*. (2007) 92:414–7. doi: 10.3324/haematol.10570
88. Zeidan AM, Forde PM, Symons H, Chen A, Smith BD, Pratz K, et al. HLA-haploidentical donor lymphocyte infusions for patients with relapsed hematologic malignancies after related HLA-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant*. (2014) 20:314–8. doi: 10.1016/j.bbmt.2013.11.020
89. Ghiso A, Raiola AM, Gualandi F, Dominietto A, Varaldo R, Van Lint MT, et al. DLI after haploidentical BMT with post-transplant CY. *Bone Marrow Transplant*. (2015) 50:56–61. doi: 10.1038/bmt.2014.217
90. Huang X-J, Liu D-H, Liu K-Y, Xu L-P, Chen Y-H, Wang Y, et al. Modified donor lymphocyte infusion after HLA-mismatched/haploidentical T cell-replete hematopoietic stem cell transplantation for prophylaxis of relapse of leukemia in patients with advanced leukemia. *J Clin Immunol*. (2008) 28:276–83. doi: 10.1007/s10875-007-9166-z
91. Jedlickova Z, Schmid C, Koenecke C, Hertenstein B, Baurmann H, Schwerdtfeger R, et al. Long-term results of adjuvant donor lymphocyte transfusion in AML after allogeneic stem cell transplantation. *Bone Marrow Transplant*. (2016) 51:663–7. doi: 10.1038/bmt.2015.234
92. Schmid C, Labopin M, Schaap N, Veelken H, Schleuning M, Stadler M, et al. Prophylactic donor lymphocyte infusion after allogeneic stem cell transplantation in acute leukaemia - a matched pair analysis by the acute leukaemia working party of EBMT. *Br J Haematol*. (2019) 184:782–7. doi: 10.1111/bjh.15691
93. Rettinger E, Willasch AM, Kreyenberg H, Borkhardt A, Holter W, Kremens B, et al. Preemptive immunotherapy in childhood acute myeloid leukemia for patients showing evidence of mixed chimerism after allogeneic stem cell transplantation. *Blood*. (2011) 118:5681–8. doi: 10.1182/blood-2011-04-348805
94. Yan C-H, Liu D-H, Liu K-Y, Xu L-P, Liu Y-R, Chen H, et al. Risk stratification-directed donor lymphocyte infusion could reduce relapse of standard-risk acute leukemia patients after allogeneic hematopoietic stem cell transplantation. *Blood*. (2012) 119:3256–62. doi: 10.1182/blood-2011-09-380386
95. Mo X-D, Zhang X-H, Xu L-P, Wang Y, Yan C-H, Chen H, et al. Comparison of outcomes after donor lymphocyte infusion with or without prior chemotherapy for minimal residual disease in acute leukemia/myelodysplastic syndrome after allogeneic hematopoietic stem cell transplantation. *Ann Hematol*. (2017) 96:829–38. doi: 10.1007/s00277-017-2960-7
96. Schmid C, Labopin M, Nagler A, Bornhäuser M, Finke J, Fassas A, et al. Donor lymphocyte infusion in the treatment of first hematological relapse after allogeneic stem-cell transplantation in adults with acute myeloid leukemia: a retrospective risk factors analysis and comparison with other strategies by the EBMT acute leukemia working party. *J Clin Oncol*. (2007) 25:4938–45. doi: 10.1200/JCO.2007.11.6053
97. Roux C, Tifratene K, Socié G, Galambrun C, Bertrand Y, Rialland F, et al. Outcome after failure of allogeneic hematopoietic stem cell transplantation in children with acute leukemia: a study by the société francophone de greffe de moelle et de thérapie cellulaire (SFGM-TC). *Bone Marrow Transplant*. (2017) 52:678–82. doi: 10.1038/bmt.2016.360
98. Miyamoto T, Fukuda T, Nakashima M, Henzan T, Kusakabe S, Kobayashi N, et al. Donor Lymphocyte infusion for relapsed hematological malignancies after unrelated allogeneic bone marrow transplantation facilitated by the Japan marrow donor program. *Biol Blood Marrow Transplant*. (2017) 23:938–44. doi: 10.1016/j.bbmt.2017.02.012
99. Michallet M, Tanguy ML, Socié G, Thiébaut A, Belhabri A, Milpied N, et al. Second allogeneic haematopoietic stem cell transplantation in relapsed acute and chronic leukaemias for patients who underwent a first allogeneic bone marrow transplantation: a survey of the société française de greffe de moelle (SFGM). *Br J Haematol*. (2000) 108:400–7. doi: 10.1046/j.1365-2141.2000.01851.x
100. Radich JP, Sanders JE, Buckner CD, Martin PJ, Petersen FB, Bensinger W, et al. Second allogeneic marrow transplantation for patients with recurrent leukemia after initial transplant with total-body irradiation-containing regimens. *J Clin Oncol*. (1993) 11:304–13. doi: 10.1200/JCO.1993.11.2.304
101. Ghosh A, Smith M, James SE, Davila ML, Velardi E, Argyropoulos KV, et al. Donor CD19 CAR T cells exert potent graft-vs.-lymphoma

activity with diminished graft-vs.-host activity. *Nat Med*. (2017) 23:242–9. doi: 10.1038/nm.4258
102. Smith M, Zakrzewski J, James S, Sadelain M. Posttransplant chimeric antigen receptor therapy. *Blood*. (2018) 131:1045–52. doi: 10.1182/blood-2017-08-752121
103. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med*. (2011) 365:725–33. doi: 10.1056/NEJMoa1103849
104. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, S et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood*. (2012) 119:2709–20. doi: 10.1182/blood-2011-10-384388
105. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med*. (2013) 368:1509–18. doi: 10.1056/NEJMoa1215134
106. Schuster SJ, Svoboda J, Chong EA, Nasta SD, Mato AR, Anak Ö, et al. Chimeric antigen receptor T cells in refractory B-cell lymphomas. *N Engl J Med*. (2017) 377:2545–54. doi: 10.1056/NEJMoa1708566
107. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, McGuirk JP, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *N Engl J Med*. (2019) 380:45–56. doi: 10.1056/NEJMoa1804980
108. Park JH, Rivière I, Gonen M, Wang X, Sénéchal B, Curran KJ, et al. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N Engl J Med*. (2018) 378:449–59. doi: 10.1056/NEJMoa1709919
109. Kochenderfer JN, Dudley ME, Carpenter RO, Kassim SH, Rose JJ, Telford WG, et al. Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood*. (2013) 122:4129–39. doi: 10.1182/blood-2013-08-519413
110. Brudno JN, Somerville RPT, Shi V, Rose JJ, Halverson DC, Fowler DH, et al. Allogeneic T cells that express an anti-CD19 chimeric antigen receptor induce remissions of B-cell malignancies that progress after allogeneic hematopoietic stem-cell transplantation without causing graft-vs.-host disease. *J Clin Oncol*. (2016) 34:1112–21. doi: 10.1200/JCO.2015.64.5929
111. Kebriaei P, Singh H, Huls MH, Figliola MJ, Bassett R, Olivares S, et al. Phase I trials using sleeping beauty to generate CD19-specific CAR T cells. *J Clin Invest*. (2016) 126:3363–76. doi: 10.1172/JCI86721
112. Jia H, Wang Z, Wang Y, Liu Y, Dai H, Tong C, et al. Haploidentical CD19/CD22 bispecific CAR-T cells induced MRD-negative remission in a patient with relapsed and refractory adult B-ALL after haploidentical hematopoietic stem cell transplantation. *J Hematol Oncol*. (2019) 12:57. doi: 10.1186/s13045-019-0741-6
113. Hu Y, Wang J, Wei G, Yu J, Luo Y, Shi J, et al. A retrospective comparison of allogenic and autologous chimeric antigen receptor T cell therapy targeting CD19 in patients with relapsed/refractory acute lymphoblastic leukemia. *Bone Marrow Transplant*. (2019) 54:1208–17. doi: 10.1038/s41409-018-0403-2
114. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJC, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*. (2017) 543:113–7. doi: 10.1038/nature21405
115. Rotolo A, Caputo VS, Holubova M, Baxan N, Dubois O, Chaudhry MS, et al. Enhanced anti-lymphoma activity of CAR19-iNKT cells underpinned by dual CD19 and CD1d targeting. *Cancer Cell*. (2018) 34:596–610.e11. doi: 10.1016/j.ccell.2018.08.017
116. Liu E, Tong Y, Dotti G, Shaim H, Savoldo B, Mukherjee M, et al. Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. *Leukemia*. (2018) 32:520–31. doi: 10.1038/leu.2017.226
117. Offner S, Hofmeister R, Romaniuk A, Kufer P, Baeuerle PA. Induction of regular cytolytic T cell synapses by bispecific single-chain antibody constructs on MHC class I-negative tumor cells. *Mol Immunol*. (2006) 43:763–71. doi: 10.1016/j.molimm.2005.03.007
118. Zhukovsky EA, Morse RJ, Maus MV. Bispecific antibodies and CARs: generalized immunotherapeutics harnessing T cell redirection. *Curr Opin Immunol*. (2016) 40:24–35. doi: 10.1016/j.coi.2016.02.006
119. Haas C, Krinner E, Brischwein K, Hoffmann P, Lutterbüse R, Schlereth B, et al. Mode of cytotoxic action of T cell-engaging BiTE antibody MT110. *Immunobiology*. (2009) 214:441–53. doi: 10.1016/j.imbio.2008.11.014
120. Hornig N, Reinhardt K, Kermer V, Kontermann RE, Müller D. Evaluating combinations of costimulatory antibody-ligand fusion proteins for targeted cancer immunotherapy. *Cancer Immunol Immunother*. (2013) 62:1369–80. doi: 10.1007/s00262-013-1441-7
121. Hornig N, Kermer V, Frey K, Diebolder P, Kontermann RE, Müller D. Combination of a bispecific antibody and costimulatory antibody-ligand fusion proteins for targeted cancer immunotherapy. *J Immunother*. (2012) 35:418–29. doi: 10.1097/CJI.0b013e3182594387
122. Arndt C, Feldmann A, von Bonin M, Cartellieri M, Ewen E-M, Koristka S, et al. Costimulation improves the killing capability of T cells redirected to tumor cells expressing low levels of CD33: description of a novel modular targeting system. *Leukemia*. (2014) 28:59–69. doi: 10.1038/leu.2013.243
123. Köhnke T, Krupka C, Tischer J, Knösel T, Subklewe M. Increase of PD-L1 expressing B-precursor ALL cells in a patient resistant to the CD19/CD3-bispecific T cell engager antibody blinatumomab. *J Hematol Oncol*. (2015) 8:111. doi: 10.1186/s13045-015-0213-6
124. Laszlo GS, Gudgeon CJ, Harrington KH, Walter RB. T-cell ligands modulate the cytolytic activity of the CD33/CD3 BiTE antibody construct, AMG 330. *Blood Cancer J*. (2015) 5:e340. doi: 10.1038/bcj.2015.68
125. Yano H, Thakur A, Tomaszewski EN, Choi M, Deol A, Lum LG. Ipilimumab augments antitumor activity of bispecific antibody-armed T cells. *J Transl Med*. (2014) 12:191. doi: 10.1186/1479-5876-12-191
126. Frikeche J, Clavert A, Delaunay J, Brissot E, Grégoire M, Gaugler B, et al. Impact of the hypomethylating agent 5-azacytidine on dendritic cells function. *Exp Hematol*. (2011) 39:1056–63. doi: 10.1016/j.exphem.2011.08.004
127. Pinto A, Maio M, Attadia V, Zappacosta S, Cimino R. Modulation of HLA-DR antigens expression in human myeloid leukaemia cells by cytarabine and 5-aza-2'-deoxycytidine. *Lancet*. (1984) 2:867–8. doi: 10.1016/S0140-6736(84)90900-0
128. Flotho C, Claus R, Batz C, Schneider M, Sandrock I, Ihde S, et al. The DNA methyltransferase inhibitors azacitidine, decitabine and zebularine exert differential effects on cancer gene expression in acute myeloid leukemia cells. *Leukemia*. (2009) 23:1019–28. doi: 10.1038/leu.2008.397
129. Atanackovic D, Luetkens T, Kloth B, Fuchs G, Cao Y, Hildebrandt Y, et al. Cancer-testis antigen expression and its epigenetic modulation in acute myeloid leukemia. *Am J Hematol*. (2011) 86:918–22. doi: 10.1002/ajh.22141
130. Goodyear O, Agathanggelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, et al. Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. *Blood*. (2010) 116:1908–18. doi: 10.1182/blood-2009-11-249474
131. Goodyear OC, Dennis M, Jilani NY, Loke J, Siddique S, Ryan G, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). *Blood*. (2012) 119:3361–9. doi: 10.1182/blood-2011-09-377044
132. Sánchez-Abarca LI, Gutierrez-Cosio S, Santamaría C, Caballero-Velazquez T, Blanco B, Herrero-Sánchez C, et al. Immunomodulatory effect of 5-azacytidine. (5-azaC): potential role in the transplantation setting. *Blood*. (2010) 115:107–21. doi: 10.1182/blood-2009-03-210393
133. Almstedt M, Blagitko-Dorfs N, Duque-Afonso J, Karbach J, Pfeifer D, Jäger E, et al. The DNA demethylating agent 5-aza-2'-deoxycytidine induces expression of NY-ESO-1 and other cancer/testis antigens in myeloid leukemia cells. *Leuk Res*. (2010) 34:899–905. doi: 10.1016/j.leukres.2010.02.004
134. Guo ZS, Hong JA, Irvine KR, Chen GA, Spiess PJ, Liu Y, et al. *De novo* induction of a cancer/testis antigen by 5-aza-2'-deoxycytidine augments adoptive immunotherapy in a murine tumor model. *Cancer Res*. (2006) 66:1105–13. doi: 10.1158/0008-5472.CAN-05-3020
135. Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, et al. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. *Cancer Res*. (1994) 54:1766–71.
136. Dubovsky JA, McNeel DG, Powers JJ, Gordon J, Sotomayor EM, Pinilla-Ibarz JA. Treatment of chronic lymphocytic leukemia with a hypomethylating

agent induces expression of NXF2, an immunogenic cancer testis antigen. *Clin Cancer Res*. (2009) 15:3406–15. doi: 10.1158/1078-0432.CCR-08-2099
137. Fonsatti E, Nicolay HJM, Sigalotti L, Calabrò L, Pezzani L, Colizzi F, et al. Functional up-regulation of human leukocyte antigen class I antigens expression by 5-aza-2'-deoxycytidine in cutaneous melanoma: immunotherapeutic implications. *Clin Cancer Res*. (2007) 13:3333–8. doi: 10.1158/1078-0432.CCR-06-3091
138. Chiappinelli KB, Strissel PL, Desrichard A, Li H, Henke C, Akman B, et al. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. *Cell*. (2015) 162:974–86. doi: 10.1016/j.cell.2015.07.011
139. Roulois D, Loo Yau H, Singhania R, Wang Y, Danesh A, Shen SY, et al. DNA-Demethylating agents target colorectal cancer cells by inducing viral mimicry by endogenous transcripts. *Cell*. (2015) 162:961–73. doi: 10.1016/j.cell.2015.07.056
140. Parker BS, Rautela J, Hertzog PJ. Antitumour actions of interferons: implications for cancer therapy. *Nat Rev Cancer*. (2016) 16:131–44. doi: 10.1038/nrc.2016.14
141. Yang H, Bueso-Ramos C, DiNardo C, Estecio MR, Davanlou M, Geng Q-R, et al. Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents. *Leukemia*. (2014) 28:1280–8. doi: 10.1038/leu.2013.355
142. Ørskov AD, Treppendahl MB, Skovbo A, Holm MS, Friis LS, Hokland M, et al. Hypomethylation and up-regulation of PD-1 in T cells by azacytidine in MDS/AML patients: a rationale for combined targeting of PD-1 and DNA methylation. *Oncotarget*. (2015) 6:9612–26. doi: 10.18632/oncotarget.3324
143. Jabbour E, Giralt S, Kantarjian H, Garcia-Manero G, Jagasia M, Kebriaei P, et al. Low-dose azacitidine after allogeneic stem cell transplantation for acute leukemia. *Cancer*. (2009) 115:1899–905. doi: 10.1002/cncr.24198
144. Schroeder T, Czibere A, Platzbecker U, Bug G, Uharek L, Luft T, et al. Azacitidine and donor lymphocyte infusions as first salvage therapy for relapse of AML or MDS after allogeneic stem cell transplantation. *Leukemia*. (2013) 27:1229–35. doi: 10.1038/leu.2013.7
145. Schroeder T, Rachlis E, Bug G, Stelljes M, Klein S, Steckel NK, Wet al. Treatment of acute myeloid leukemia or myelodysplastic syndrome relapse after allogeneic stem cell transplantation with azacitidine and donor lymphocyte infusions–a retrospective multicenter analysis from the German cooperative transplant study group. *Biol Blood Marrow Transplant*. (2015) 21:653–60. doi: 10.1016/j.bbmt.2014.12.016
146. Craddock C, Labopin M, Robin M, Finke J, Chevallier P, Yakoub-Agha I, et al. Clinical activity of azacitidine in patients who relapse after allogeneic stem cell transplantation for acute myeloid leukemia. *Haematologica*. (2016) 101:879–83. doi: 10.3324/haematol.2015.140996
147. Ghobadi A, Choi J, Fiala MA, Fletcher T, Liu J, Eissenberg LG, et al. Phase I study of azacitidine following donor lymphocyte infusion for relapsed acute myeloid leukemia post allogeneic stem cell transplantation. *Leuk Res*. (2016) 49:1–6. doi: 10.1016/j.leukres.2016.07.010
148. Woo J, Deeg HJ, Storer B, Yeung C, Fang M, Mielcarek M, et al. Factors determining responses to azacitidine in patients with myelodysplastic syndromes and acute myeloid leukemia with early post-transplantation relapse: a prospective trial. *Biol Blood Marrow Transplant*. (2017) 23:176–9. doi: 10.1016/j.bbmt.2016.10.016
149. Choi SW, Braun T, Chang L, Ferrara JLM, Pawarode A, Magenau JM, et al. Vorinostat plus tacrolimus and mycophenolate to prevent graft-vs.-host disease after related-donor reduced-intensity conditioning allogeneic haemopoietic stem-cell transplantation: a phase 1/2 trial. *Lancet Oncol*. (2014) 15:87–95. doi: 10.1016/S1470-2045(13)70512-6
150. Choi SW, Gatza E, Hou G, Sun Y, Whitfield J, Song Y, et al. Histone deacetylase inhibition regulates inflammation and enhances tregs after allogeneic hematopoietic cell transplantation in humans. *Blood*. (2015) 125:815–9. doi: 10.1182/blood-2014-10-605238
151. Bug G, Burchert A, Wagner E-M, Kröger N, Berg T, Güller S, et al. Phase I/II study of the deacetylase inhibitor panobinostat after allogeneic stem cell transplantation in patients with high-risk MDS or AML (PANOBEST trial). *Leukemia*. (2017) 31:2523–5. doi: 10.1038/leu.2017.242
152. Cornelissen JJ, van Norden Y, van Gelder M, Breems DA, Maertens J, Jongen-Lavrencic M, et al. Early post-transplant epigenetic therapy by panobinostat and decitabine followed by Donor Lymphocyte Infusion (DLI): interim results of the HOVON-116 phase I/II feasibility study in poor-risk AML recipients of allogeneic stem cell transplantation (alloHSCT). *Blood J*. (2016) 128:832. doi: 10.1182/blood.V128.22.832.832
153. Greil R, Hutterer E, Hartmann TN, Pleyer L. Reactivation of dormant anti-tumor immunity - a clinical perspective of therapeutic immune checkpoint modulation. *Cell Commun Signal*. (2017) 15:5. doi: 10.1186/s12964-016-0155-9
154. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. (2012) 12:252–64. doi: 10.1038/nrc3239
155. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. (2013) 13:227–42. doi: 10.1038/nri3405
156. Bashey A, Medina B, Corringham S, Pasek M, Carrier E, Vrooman L, et al. CTLA4 blockade with ipilimumab to treat relapse of malignancy after allogeneic hematopoietic cell transplantation. *Blood*. (2009) 113:1581–8. doi: 10.1182/blood-2008-07-168468
157. Davids MS, Kim HT, Bachireddy P, Costello C, Liguori R, Savell A, et al. Ipilimumab for patients with relapse after allogeneic transplantation. *N Engl J Med*. (2016) 375:143–53. doi: 10.1056/NEJMoa1601202
158. Albring JC, Inselmann S, Sauer T, Schliemann C, Altvater B, Kailayangiri S, et al. PD-1 checkpoint blockade in patients with relapsed AML after allogeneic stem cell transplantation. *Bone Marrow Transplant*. (2017) 52:317–20. doi: 10.1038/bmt.2016.274
159. McDuffee E, Aue G, Cook L, Ramos-Delgado C, Shalabi R, Worthy T, et al. Tumor regression concomitant with steroid-refractory GvHD highlights the pitfalls of PD-1 blockade following allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. (2017) 52:759–61. doi: 10.1038/bmt.2016.346
160. Onizuka M, Kojima M, Matsui K, Machida S, Toyosaki M, Aoyama Y, et al. Successful treatment with low-dose nivolumab in refractory hodgkin lymphoma after allogeneic stem cell transplantation. *Int J Hematol*. (2017) 106:141–5. doi: 10.1007/s12185-017-2181-9
161. Herbaux C, Gauthier J, Brice P, Drumez E, Ysebaert L, Doyen H, et al. Efficacy and tolerability of nivolumab after allogeneic transplantation for relapsed hodgkin lymphoma. *Blood*. (2017) 129:2471–8. doi: 10.1182/blood-2016-11-749556
162. Haverkos BM, Abbott D, Hamadani M, Armand P, Flowers ME, Merryman R, et al. PD-1 blockade for relapsed lymphoma post-allogeneic hematopoietic cell transplant: high response rate but frequent GVHD. *Blood*. (2017) 130:221–8. doi: 10.1182/blood-2017-01-761346
163. Wang L, Amoozgar Z, Huang J, Saleh MH, Xing D, Orsulic S, et al. Decitabine enhances lymphocyte migration and function and synergizes with CTLA-4 blockade in a murine ovarian cancer model. *Cancer Immunol Res*. (2015) 3:1030–41. doi: 10.1158/2326-6066.CIR-15-0073
164. Daver N, Boddu P, Garcia-Manero G, Yadav SS, Sharma P, Allison J, et al. Hypomethylating agents in combination with immune checkpoint inhibitors in acute myeloid leukemia and myelodysplastic syndromes. *Leukemia*. (2018) 32:1094–105. doi: 10.1038/s41375-018-0070-8
165. Dear AE. Epigenetic modulators and the new immunotherapies. *N Engl J Med*. (2016) 374:684–6. doi: 10.1056/NEJMcibr1514673
166. Daver N, Garcia-Manero G, Basu S, Boddu PC, Alfayez M, Cortes JE, et al. Efficacy, safety, and biomarkers of response to azacitidine and nivolumab in relapsed/refractory acute myeloid leukemia: a nonrandomized, open-label, phase II study. *Cancer Discov*. (2019) 9:370–83. doi: 10.1158/2159-8290.CD-18-0774
167. Ettinghausen SE, Lipford EH, Mulé JJ, Rosenberg SA. Recombinant interleukin 2 stimulates *in vivo* proliferation of adoptively transferred lymphokine-activated killer (LAK) cells. *J Immunol*. (1985) 135:3623–35.
168. Alatrash G, Jakher H, Stafford PD, Mittendorf EA. Cancer immunotherapies, their safety and toxicity. *Expert Opin Drug Saf*. (2013) 12:631–45. doi: 10.1517/14740338.2013.795944
169. Foa R, Guarini A, Gansbacher B. IL2 treatment for cancer: from biology to gene therapy. *Br J Cancer*. (1992) 66:992–8. doi: 10.1038/bjc.1992.400
170. Buyse M, Squifflet P, Lange BJ, Alonzo TA, Larson RA, Kolitz JE, et al. Individual patient data meta-analysis of randomized trials evaluating IL-2 monotherapy as remission maintenance therapy in acute myeloid leukemia. *Blood*. (2011) 117:7007–13. doi: 10.1182/blood-2011-02-337725

171. Acuto O, Di Bartolo V, Michel F. Tailoring T-cell receptor signals by proximal negative feedback mechanisms. *Nat Rev Immunol*. (2008) 8:699–712. doi: 10.1038/nri2397
172. Ustun C, Miller JS, Munn DH, Weisdorf DJ, Blazar BR. Regulatory T cells in acute myelogenous leukemia: is it time for immunomodulation? *Blood*. (2011) 118:5084–95. doi: 10.1182/blood-2011-07-365817
173. Kolitz JE, George SL, Benson DM, Maharry K, Marcucci G, Vij R, et al. Recombinant interleukin-2 in patients aged younger than 60 years with acute myeloid leukemia in first complete remission: results from cancer and leukemia group B 19808. *Cancer*. (2014) 120:1010–7. doi: 10.1002/cncr.28516
174. Platanias LC. Interferons and their antitumor properties. *J Interferon Cytokine Res*. (2013) 33:143–4. doi: 10.1089/jir.2013.0019
175. Le Bon A, Tough DF. Type I interferon as a stimulus for cross-priming. *Cytokine Growth Factor Rev*. (2008) 19:33–40. doi: 10.1016/j.cytogfr.2007.10.007
176. Papewalis C, Jacobs B, Wuttke M, Ullrich E, Baehring T, Fenk R, et al. IFN-alpha skews monocytes into CD56+-expressing dendritic cells with potent functional activities *in vitro* and *in vivo*. *J Immunol*. (2008) 180:1462–70. doi: 10.4049/jimmunol.180.3.1462
177. Bekisz J, Sato Y, Johnson C, Husain SR, Puri RK, Zoon KC. Immunomodulatory effects of interferons in malignancies. *J Interferon Cytokine Res*. (2013) 33:154–61. doi: 10.1089/jir.2012.0167
178. Anguille S, Lion E, Willemen Y, Van Tendeloo VFI, Berneman ZN, Smits ELJM. Interferon-α in acute myeloid leukemia: an old drug revisited. *Leukemia*. (2011) 25:739–48. doi: 10.1038/leu.2010.324
179. Colamonici OR, Domanski P, Platanias LC, Diaz MO. Correlation between interferon (IFN) alpha resistance and deletion of the IFN alpha/beta genes in acute leukemia cell lines suggests selection against the IFN system. *Blood*. (1992) 80:744–9. doi: 10.1182/blood.V80.3.744.bloodjournal803744
180. Smits ELJM, Anguille S, Berneman ZN. Interferon α may be back on track to treat acute myeloid leukemia. *Oncoimmunology*. (2013) 2:e23619. doi: 10.4161/onci.23619
181. Robb RJ, Kreijveld E, Kuns RD, Wilson YA, Olver SD, Don ALJ, et al. Type I-IFNs control GVHD and GVL responses after transplantation. *Blood*. (2011) 118:3399–409. doi: 10.1182/blood-2010-12-325746
182. Steel JC, Waldmann TA, Morris JC. Interleukin-15 biology and its therapeutic implications in cancer. *Trends Pharmacol Sci*. (2012) 33:35–41. doi: 10.1016/j.tips.2011.09.004
183. Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC, et al. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol*. (2015) 33:74–82. doi: 10.1200/JCO.2014.57.3329
184. Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, et al. IL-15 enhances the *in vivo* antitumor activity of tumor-reactive CD8+ T cells. *Proc Natl Acad Sci USA*. (2004) 101:1969–74. doi: 10.1073/pnas.0307298101
185. Cieri N, Oliveira G, Greco R, Forcato M, Taccioli C, Cianciotti B, et al. Generation of human memory stem T cells after haploidentical T-replete hematopoietic stem cell transplantation. *Blood*. (2015) 125:2865–74. doi: 10.1182/blood-2014-11-608539
186. Ring AM, Lin J-X, Feng D, Mitra S, Rickert M, Bowman GR, et al. Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat Immunol*. (2012) 13:1187–95. doi: 10.1038/ni.2449
187. Romee R, Cooley S, Berrien-Elliott MM, Westervelt P, Verneris MR, Wagner JE, et al. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. *Blood*. (2018) 131:2515–27. doi: 10.1182/blood-2017-12-823757
188. Escobar G, Barbarossa L, Barbiera G, Norelli M, Genua M, Ranghetti A, et al. Interferon gene therapy reprograms the leukemia microenvironment inducing protective immunity to multiple tumor antigens. *Nat Commun*. (2018) 9:2896. doi: 10.1038/s41467-018-05315-0
189. Pastor F, Berraondo P, Etxeberria I, Frederick J, Sahin U, Gilboa E, et al. An RNA toolbox for cancer immunotherapy. *Nat Rev Drug Discov*. (2018) 17:751–67. doi: 10.1038/nrd.2018.132
190. Olavarria E, Siddique S, Griffiths MJ, Avery S, Byrne JL, Piper KP, et al. Posttransplantation imatinib as a strategy to postpone the requirement for immunotherapy in patients undergoing reduced-intensity allografts for chronic myeloid leukemia. *Blood*. (2007) 110:4614–7. doi: 10.1182/blood-2007-04-082990
191. Ribera J-M, Oriol A, González M, Vidriales B, Brunet S, Esteve J, et al. Concurrent intensive chemotherapy and imatinib before and after stem cell transplantation in newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia. final results of the CSTIBES02 trial. *Haematologica*. (2010) 95:87–95. doi: 10.3324/haematol.2009.011221
192. Shin JY, Hu W, Naramura M, Park CY. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J Exp Med*. (2014) 211:217–31. doi: 10.1084/jem.20131128
193. Bodine DM, Seidel NE, Zsebo KM, Orlic D. *In vivo* administration of stem cell factor to mice increases the absolute number of pluripotent hematopoietic stem cells. *Blood*. (1993) 82:445–55. doi: 10.1182/blood.V82.2.445.445
194. Thorén LA, Liuba K, Bryder D, Nygren JM, Jensen CT, Qian H, et al. Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol*. (2008) 180:2045–53. doi: 10.4049/jimmunol.180.4.2045
195. Zitvogel L, Rusakiewicz S, Routy B, Ayyoub M, Kroemer G. Immunological off-target effects of imatinib. *Nat Rev Clin Oncol*. (2016) 13:431–46. doi: 10.1038/nrclinonc.2016.41
196. Appel S, Rupf A, Weck MM, Schoor O, Brümmendorf TH, Weinschenk T, et al. Effects of imatinib on monocyte-derived dendritic cells are mediated by inhibition of nuclear factor-kappaB and Akt signaling pathways. *Clin Cancer Res*. (2005) 11:1928–40. doi: 10.1158/1078-0432.CCR-04-1713
197. Appel S, Boehmler AM, Grünebach F, Müller MR, Rupf A, Weck MM, et al. Imatinib mesylate affects the development and function of dendritic cells generated from CD34+ peripheral blood progenitor cells. *Blood*. (2004) 103:538–44. doi: 10.1182/blood-2003-03-0975
198. Taïeb J, Maruyama K, Borg C, Terme M, Zitvogel L. Imatinib mesylate impairs Flt3L-mediated dendritic cell expansion and antitumor effects *in vivo*. *Blood*. (2004) 103:1966–7. doi: 10.1182/blood-2003-10-3475
199. Boissel N, Rousselot P, Raffoux E, Cayuela J-M, Maarek O, Charron D, et al. Defective blood dendritic cells in chronic myeloid leukemia correlate with high plasmatic VEGF and are not normalized by imatinib mesylate. *Leukemia*. (2004) 18:1656–61. doi: 10.1038/sj.leu.2403474
200. van Dongen M, Savage NDL, Jordanova ES, Briaire-de Bruijn IH, Walburg KV, Ottenhoff THM, et al. Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. *Int J Cancer*. (2010) 127:899–909. doi: 10.1002/ijc.25113
201. Cavnar MJ, Zeng S, Kim TS, Sorenson EC, Ocuin LM, Balachandran VP, et al. KIT oncogene inhibition drives intratumoral macrophage M2 polarization. *J Exp Med*. (2013) 210:2873–86. doi: 10.1084/jem.20130875
202. Seggewiss R, Loré K, Greiner E, Magnusson MK, Price DA, Douek DC, et al. Imatinib inhibits T-cell receptor-mediated T-cell proliferation and activation in a dose-dependent manner. *Blood*. (2005) 105:2473–9. doi: 10.1182/blood-2004-07-2527
203. Gao H, Lee B-N, Talpaz M, Donato NJ, Cortes JE, Kantarjian HM, et al. Imatinib mesylate suppresses cytokine synthesis by activated CD4 T cells of patients with chronic myelogenous leukemia. *Leukemia*. (2005) 19:1905–11. doi: 10.1038/sj.leu.2403933
204. de Lavallade H, Khoder A, Hart M, Sarvaria A, Sekine T, Alsuliman A, et al. Tyrosine kinase inhibitors impair B-cell immune responses in CML through off-target inhibition of kinases important for cell signaling. *Blood*. (2013) 122:227–38. doi: 10.1182/blood-2012-11-465039
205. Steegmann JL, Moreno G, Aláez C, Osorio S, Granda A, de la Cámara R, et al. Chronic myeloid leukemia patients resistant to or intolerant of interferon alpha and subsequently treated with imatinib show reduced immunoglobulin levels and hypogammaglobulinemia. *Haematologica*. (2003) 88:762–8.
206. Carulli G, Cannizzo E, Ottaviano V, Cervetti G, Buda G, Galimberti S, et al. Abnormal phenotype of bone marrow plasma cells in patients with chronic myeloid leukemia undergoing therapy with imatinib. *Leuk Res*. (2010) 34:1336–9. doi: 10.1016/j.leukres.2010.01.012
207. Balachandran VP, Cavnar MJ, Zeng S, Bamboat ZM, Ocuin LM, Obaid H, et al. Imatinib potentiates antitumor T cell responses in gastrointestinal

stromal tumor through the inhibition of ido. *Nat Med*. (2011) 17:1094–100. doi: 10.1038/nm.2438

208. Larmonier N, Janikashvili N, LaCasse CJ, Larmonier CB, Cantrell J, Situ E, et al. Imatinib mesylate inhibits CD4+ CD25+ regulatory T cell activity and enhances active immunotherapy against BCR-ABL- tumors. *J Immunol*. (2008) 181:6955–63. doi: 10.4049/jimmunol.181.10.6955
209. Giallongo C, Parrinello N, Tibullo D, La Cava P, Romano A, Chiarenza A, et al. Myeloid derived suppressor cells (MDSCs) are increased and exert immunosuppressive activity together with polymorphonuclear leukocytes (PMNs) in chronic myeloid leukemia patients. *PLoS ONE*. (2014) 9:e101848. doi: 10.1371/journal.pone.0101848
210. Christiansson L, Söderlund S, Mangsbo S, Hjorth-Hansen H, Höglund M, Markevärn B, et al. The tyrosine kinase inhibitors imatinib and dasatinib reduce myeloid suppressor cells and release effector lymphocyte responses. *Mol Cancer Ther*. (2015) 14:1181–91. doi: 10.1158/1535-7163.MCT-14-0849
211. Christiansson L, Söderlund S, Svensson E, Mustjoki S, Bengtsson M, Simonsson B, et al. Increased level of myeloid-derived suppressor cells, programmed death receptor ligand 1/programmed death receptor 1, and soluble CD25 in Sokal high risk chronic myeloid leukemia. *PLoS ONE*. (2013) 8:e55818. doi: 10.1371/journal.pone.0055818
212. Legros L, Bourcier C, Jacquel A, Mahon F-X, Cassuto J-P, Auberger P, et al. Imatinib mesylate (STI571) decreases the vascular endothelial growth factor plasma concentration in patients with chronic myeloid leukemia. *Blood*. (2004) 104:495–501. doi: 10.1182/blood-2003-08-2695
213. Legros L, Guilhot J, Huault S, Mahon FX, Preudhomme C, Guilhot F, et al. Interferon decreases VEGF levels in patients with chronic myeloid leukemia treated with imatinib. *Leuk Res*. (2014) 38:662–5. doi: 10.1016/j.leukres.2014.01.010
214. Chen CI-U, Maecker HT, Lee PP. Development and dynamics of robust T-cell responses to CML under imatinib treatment. *Blood*. (2008) 111:5342–9. doi: 10.1182/blood-2007-12-128397
215. Riva G, Luppi M, Barozzi P, Quadrelli C, Basso S, Vallerini D, et al. Emergence of BCR-ABL-specific cytotoxic T cells in the bone marrow of patients with Ph+ acute lymphoblastic leukemia during long-term imatinib mesylate treatment. *Blood*. (2010) 115:1512–8. doi: 10.1182/blood-2009-06-230391
216. Riva G, Luppi M, Lagreca I, Barozzi P, Quadrelli C, Vallerini D, et al. Long-term molecular remission with persistence of BCR-ABL1-specific cytotoxic T cells following imatinib withdrawal in an elderly patient with philadelphia-positive ALL. *Br J Haematol*. (2014) 164:299–302. doi: 10.1111/bjh.12612
217. Ohyashiki K, Katagiri S, Tauchi T, Ohyashiki JH, Maeda Y, Matsumura I, et al. Increased natural killer cells and decreased CD3(+)CD8(+)CD62L(+) T cells in CML patients who sustained complete molecular remission after discontinuation of imatinib. *Br J Haematol*. (2012) 157:254–6. doi: 10.1111/j.1365-2141.2011.08939.x
218. Hsiao M, Martynova A, Yaghmour G, Foss C. Investigating the relationship between CD34+and CD3+ cell doses, one-year graft-vs.-relapse-free-survival, graft-vs.-host disease, and overall survival in haploidentical hematopoietic stem cell transplantation: a single center experience. *Blood*. (2019) 134:2051. doi: 10.1182/blood-2019-129647
219. Impola U, Larjo A, Salmenniemi U, Putkonen M, Itälä-Remes M, Partanen J. Graft immune cell composition associates with clinical outcome of allogeneic hematopoietic stem cell transplantation in patients with AML. *Front Immunol*. (2016) 7:523. doi: 10.3389/fimmu.2016.00523
220. Fisher SA, Lamikanra A, Dorée C, Gration B, Tsang P, Danby RD, et al. Increased regulatory T cell graft content is associated with improved outcome in haematopoietic stem cell transplantation: a systematic review. *Br J Haematol*. (2017) 176:448–63. doi: 10.1111/bjh.14433

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REVIEW
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Post-transplantation Cyclophosphamide: From HLA-Haploidentical to Matched-Related and Matched-Unrelated Donor Blood and Marrow Transplantation

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Following allogeneic blood and marrow transplantation (BMT), graft-versus-host disease (GvHD) continues to represent a significant cause of treatment failure, despite the routine use of conventional, mainly calcineurin inhibitor-based prophylaxis. Recently, post-transplant cyclophosphamide (PTCy) has emerged as a safe and efficacious alternative. First, omitting the need for *ex vivo* T-cell depletion in the setting of haploidentical transplantation, growing evidence supports PTCy role in GvHD prevention in matched-related and matched-unrelated transplants. Through improved understanding of GvHD pathophysiology and advancements in drug development, PTCy emerges as a unique opportunity to design calcineurin inhibitor-free strategies by integrating agents that target different stages of GvHD development.

Keywords: GvHD prevention, post-transplant cyclophosphamide, matched-related donor, matched unrelated donor, bortezomib, calcineurin inhibitor-free

INTRODUCTION

Despite continued improvement in the outcomes of allogeneic blood and marrow transplant (BMT) over the last decade, the prospects of acute and chronic graft-versus-host disease (aGvHD and cGvHD) continue to drive treatment-related mortality (TRM) and limit the utility and wide applicability of this valuable treatment modality (1). Conventional combinations of calcineurin (CN) or mammalian target of rapamycin (mTOR) inhibitors, coupled with either methotrexate (MTX) or mycophenolate mofetil (MMF), achieve rates of aGvHD and cGvHD of approximately 40–75% and 40–70% following matched-related donor (MRD) and matched-unrelated donor (MUD) transplants (2). In addition to their partial efficacy, these regimens target T-cells broadly and indiscriminately, therefore delaying immune reconstitution and hampering graft-versus-leukemia (GvL) effect. Furthermore, both CN inhibitors (CNI) and mTOR inhibitors (mTORI) have a narrow therapeutic index with multiple drug interactions rendering prescriber experience and patients' compliance essential for their safety and efficacy (3). Lastly,

as these agents are administered for 6 to 9 months, they typically prevent early post-transplant introduction of interventions and small molecules aimed to decrease the risk of disease relapse.

Post-transplant cyclophosphamide (PTCy), initially developed to overcome human leukocyte antigen (HLA) barriers in the setting of haploidentical transplantation (4), has proved promising following MRD and MUD transplants (5–7). Furthermore, PTCy may represent an ideal platform for the development of CN and mTORI-free GvHD prevention strategies.

MECHANISMS of PTCy In GVHD PREVENTION

Cyclophosphamide is an alkylating agent that acts through its metabolites, phosphoramide and acrolein, to induce DNA strand breakage that ultimately leads to replication stress in rapidly dividing cells (8). The efficacy of cyclophosphamide appears to be cell-cycle dependent and is highest in the G1 and S phases (9). In studies evaluating the specific effects of cyclophosphamide on cytotoxic T-cell lines, Strauss et al. observed that increased apoptosis, mediated by increased Fas expression, may differentiate cyclophosphamide from other immunosuppressive agents (4, 10, 11).

The biological underpinnings of PTCy-induced immune tolerance have yet to be fully elucidated. However, there is evidence to support that PTCy eliminates alloreactive T-cell clones of both donor and host origin in the early post-transplantation period with relative preservation of regulatory T-cells (12, 13). This is supported by early evidence from murine skin allograft experiments where allografted mice were treated with cyclophosphamide shortly after engraftment. In these test animals, donor-derived alloreactive T-cell populations were eliminated via extra-thymic mechanism, presumably related to cyclophosphamide administration (14, 15). Simultaneously, regulatory T-cells were selectively spared, possibly due to their expression of a specific aldehyde dehydrogenase that confers resistance to cyclophosphamide (16, 17). Life-long immune tolerance was subsequently maintained by central, intra-thymic clonal deletion of the anti-host T-cells derived from donor hematopoietic stem cells.

The previously demonstrated pivotal role of regulatory T-cells in PTCy-induced immune tolerance was recently corroborated by a series of experiments performed by Waschmuth et al. Mice treated with PTCy at 25 mg/kg on day +3 and +4 following haploidentical transplantation showed significantly less severe GvHD than mice treated with 5 or 100 mg/kg a day. In these experiments, immune tolerance developed despite the persistence of alloreactive T-cells following optimally dosed PTCy and in the absence of thymus. Rather than eliminating alloreactive T-cells, PTCy induced functional impairment of these cells, supported by robust suppressive mechanisms that included rapid and preferential recovery of regulatory T-cells (18, 19). Further validation of these mechanisms will improve our understanding of PTCy-induced immune tolerance and identify the optimal dosing of cyclophosphamide.

PTCy IN HAPLOIDENTICAL TRANSPLANT

An early study established that PTCy can overcome HLA barriers and omit the need for *ex vivo* T-cell depletion following a non-myeloablative preparative regimen and haploidentical bone marrow transplant (20, 21). In this trial, the investigators compared one dose of PTCy administered on day +3 and two doses of PTCy on day +3 and +4, demonstrating a decreased incidence of cGvHD in the group receiving two doses (25% versus 5%, $p = 0.05\%$) with no differences in aGvHD, event-free survival (EFS) or overall survival (OS). The incidence of grades II-IV and III-IV aGvHD for the entire cohort was 34 and 6%, respectively (21). This data was confirmed in a larger study conducted by Kasamon et al. and Munchel et al. In a cohort of 210 patients, the incidence of grades II-IV acute and cGvHD were 27 and 13%, respectively (22, 23). The rates of disease relapse, EFS, and OS were 55, 35, and 27%, respectively.

Following the initial studies, which were focused on bone marrow as the graft source, the safety and efficacy of PTCy-based GvHD prevention were validated following both myeloablative and non-myeloablative conditioning regimens by several investigators. In a study by Solomon et al. using busulfan-based myeloablative conditioning, the incidence of grade II-IV and III-IV aGvHD and cGvHD were 30, 10, and 35% (24). Similar results were reported by Bhamidipati et al. following a non-myeloablative preparative regimen (25). More recently, Wang et al. showed that the addition of low dose PTCy (14.5 mg/kg on day +3 and +4), to the so-called Beijing protocol, reduced the incidence of grade II-IV aGvHD and improved GvHD- and relapse-free survival (GRFS) (26).

Based on these studies and others, haploidentical transplantation has become, with the advent of PTCy, one of the most commonly used alternative donor strategies. Multiple retrospective comparisons have demonstrated similar overall survival of haploidentical transplantation to that of HLA-matched donor and cord blood transplants (27).

PTCy AS MONOTHERAPY IN MRD AND MUD TRANSPLANT

Since establishing its role in the haploidentical setting, several investigators examined the applicability of PTCy to GvHD prevention in MRD and MUD transplants. Luznik et al. reported the incidence of acute and cGvHD following myeloablative conditioning in 117 recipients of MRD ($n = 78$) and MUD ($n = 39$) bone marrow grafts. In this study, GvHD prophylaxis consisted of a single agent PTCy. Grades II-IV and III-IV acute GvHD rates were 43 and 10% for the entire cohort. The long-term incidence of cGvHD was particularly low at 10%. EFS sand OS were 55 and 39% (5). Interestingly, 43% of patients did not require any other form of immunosuppressive therapy (28). These favorable results were corroborated by a similar multi-institutional trial by Kankary et al. with a low incidence of cGvHD at 14% (6).

Unfortunately, when Alousi et al. examined PTCy as the only GvHD prophylaxis following reduced-intensity preparative regimen and peripheral blood grafts, the results were strikingly different (29). In this study, 38 patients received bone marrow and 11 patients received peripheral blood grafts. Twenty-two patients received rabbit anti-thymocyte globulin (rATG) before the study was amended to omit rATG. The rates of grade II-IV and grade III-IV aGVHD were of 58% and 22%, whereas the rate of cGVHD was 18%. When the authors compared the results to a matched, historical cohort of patients receiving TAC and MTX for GvHD prophylaxis, significantly higher rates of all grades of aGvHD [46% vs. 19%, hazard ratio (HR) = 2.8, p = 0.02] as well as inferior TRM (HR = 3.3, p = 0.035) and OS (HR = 1.9, p = 0.02) were observed in the PTCy cohort. There were no differences in cGvHD between the prospectively treated patients and the historical control (29). Similarly, unsatisfactory results were reported in a smaller phase II study by Holtick et al. (30). The authors examined the safety and efficacy of PTCy as monotherapy for GvHD prevention following reduced-intensity conditioning and MRD and MUD peripheral blood transplants. The rate of TRM was unacceptably high at 36%, principally attributable to an increased rate of severe intestinal aGVHD. A study by Bradstock et al. was terminated early when four out of the first five patients developed life-threatening aGvHD, two of whom died (31).

Cumulatively, the current evidence suggests that, while single-agent PTCy may represent a viable prophylactic option in patients receiving myeloablative conditioning and bone marrow graft, it is inadequate in the setting of reduced-intensity conditioning and peripheral blood transplantation.

PTCy AND CNI OR mTOR INHIBITORS IN MRD AND MUD TRANSPLANT

Given the shortcomings of PTCy as monotherapy for GvHD prophylaxis following MRD and MUD peripheral blood transplants, several groups reverted to combining PTCy with a CNI or mTORI with or without MMF, aiming to reduce the relatively high incidence of cGvHD characteristic of CNI and mTOR inhibitors-based combinations. To this end, Mierlcarek et al. combined PTCy with cyclosporine A (CSA) in patients receiving myeloablative conditioning. The rates of grades II-IV and III-IV aGvHD were favorable at 77 and 0% and again with a low incidence of cGvHD at 16%. The rates of TRM and disease relapse were 14 and 17% (32). Moiseev et al. compared the outcomes of patients receiving a combination PTCy, TAC and MMF to the outcomes of consecutive historical control patients receiving TAC, MMF and rATG following myeloablative conditioning and MUD or 1–2 HLA loci mismatched-unrelated donor peripheral blood grafts. The rates of grades II-IV and III-IV aGvHD were 19% vs. 45% (p = 0.003) and 4% vs. 27%, ($p < 0.001$), respectively. The incidence of cGvHD was 16% vs. 65% ($p < 0.001$). EFS and OS were also improved in the PTCy group (HR = 0.49, 95% CI 0.31–0.78, p = 0.006, and 0.43, 95% CI 0.26–0.7, p = 0.007) (33). Carnevale-Schianca et al. employed the same GvHD prevention regimen in 35 patients receiving RIC

TABLE 1 | Selected registered studies of PTCy-based GvHD prevention in MRD and MUD transplantation.

Study identification	Study type	Intervention	Responsible party
NCT04202835	Phase III, Randomized	rATG versus rATG and PTCy	Sarah Kleiboer
NCT04232085	Phase II	PTCy, tacrolimus, and MMF in patients with primary bone marrow failure or immunodeficiency syndromes	Orly Klein
NCT03357159	Phase II	PTCy and rATG	Arnon Nagler
NCT02629120	Phase II	PTCy and sirolimus in patients with chronic granulomatous disease	Elizabeth Kang
NCT02861417	Phase II	PTCy, tacrolimus and MMF	Uday Popat
NCT03192397	Phase II	PTCy, sirolimus and MMF	Christine Ho
NCT03818334	Phase III, Randomized	rATG, CNI, and MMF versus PTCy, CNI and MMF	Andreza Feisoa Ribeiro
NCT03945591	Phase II	PTCy, bortezomib and rATG	A Samer Al-Homsi
NCT03602898	Phase II	CNI and MTX versus CNI, MTX, and rATG or PTCy and CNI	Masumi Ueda
NCT03959241	Phase III, Randomized	Tacrolimus and MTX versus PTCy, tacrolimus and MMF (GvHD prevention and stool microbiome)	Mary Horowitz
NCT03555851	Phase I	PTCy (pharmacogenetics predictors of efficacy)	Chojecki, Aleksander
NCT03246906	Phase II	Cyclosporine, sirolimus, and MMF versus PTCy, cyclosporine, and sirolimus	Masumi Ueda
NCT03263767	Phase II	PTCy	Patrice Cehvallier
NCT04160390	Phase I	PTCy (biomarkers predictors of efficacy)	Jeannine McCune
NCT03680092	Phase II	Tacrolimus and MTX versus PTCy and abatacept	Divya Koura
NCT02556931	Phase II	PTCy, tacrolimus (short course) and MMF	Amy E. Dezern
NCT02876679	Phase II	Cyclosporine, MMF and rATG versus PTCY, cyclosporine, and MMF	Mohamad Mothy
NCT02833805	Phase II	PTCy, tacrolimus, and MMF in patients with severe aplastic anemia	Amy E. Dezern

rATG, rabbit anti-thymocyte globulin; MMF, mycophenolate mofetil; MTX, methotrexate.

and MRD, MUD, or 1 HLA locus mismatched-unrelated donor peripheral blood transplants. The patients achieved grades II-IV aGvHD and cGvHD rates of 17 and 7% with no grade IV aGvHD. The 2-year TRM rate was 3% with EFS and OS rates of 54% and 77% (34).

Two studies examined the combination of PTCy and sirolimus. Solomon et al. conducted a phase II study that included 26 patients treated with RIC following MUD and peripheral blood transplants. Sirolimus was stopped without taper between day +90 and 100. The rates of grade III-IV acute and cGvHD were higher than the aforementioned rates with PTCy and CNI combinations at 16 and 31% (35). Greco et al. elaborated on the use of PTCy in combination with sirolimus in 28 patients receiving a myeloablative preparative regimen and MRD or MUD peripheral blood allografts. MMF was added to the regimen in patients receiving MUD transplants. The incidence of grades II-IV acute and cGvHD seemed better at 23 and 13% (36).

The most substantial evidence favoring a PTCy-based GvHD prevention strategy in the setting of MRD or MUD donor transplants stems from a recent Blood and Marrow Transplant Clinical Trial Net randomized phase II trial (37). In this trial patients received RIC and were randomized to one of three GvHD prevention regimens: TAC, MTX, and bortezomib, TAC, MTX and maraviroc or PTCy, TAC, and MMF. Patients with MRD, MUD, or 1 HLA locus mismatched-unrelated donors were included. Each of the trial's three groups was then compared to a contemporaneous prospective control group receiving TAC and MTX from non-participating institutions. Comorbidities were more frequent in the control group. The distribution of the conditioning regimens was also different. Among the three groups, only the group treated with PTCy-based prophylaxis had better outcomes in comparison to the control cohort. The rates of grades II-IV and III-IV aGvHD for the PTCy group were 27% (90% CI 20%–35%) and 2% (90% CI 0–5%). The corresponding rates in the control group were 30% (90% CI 25%–36%) and

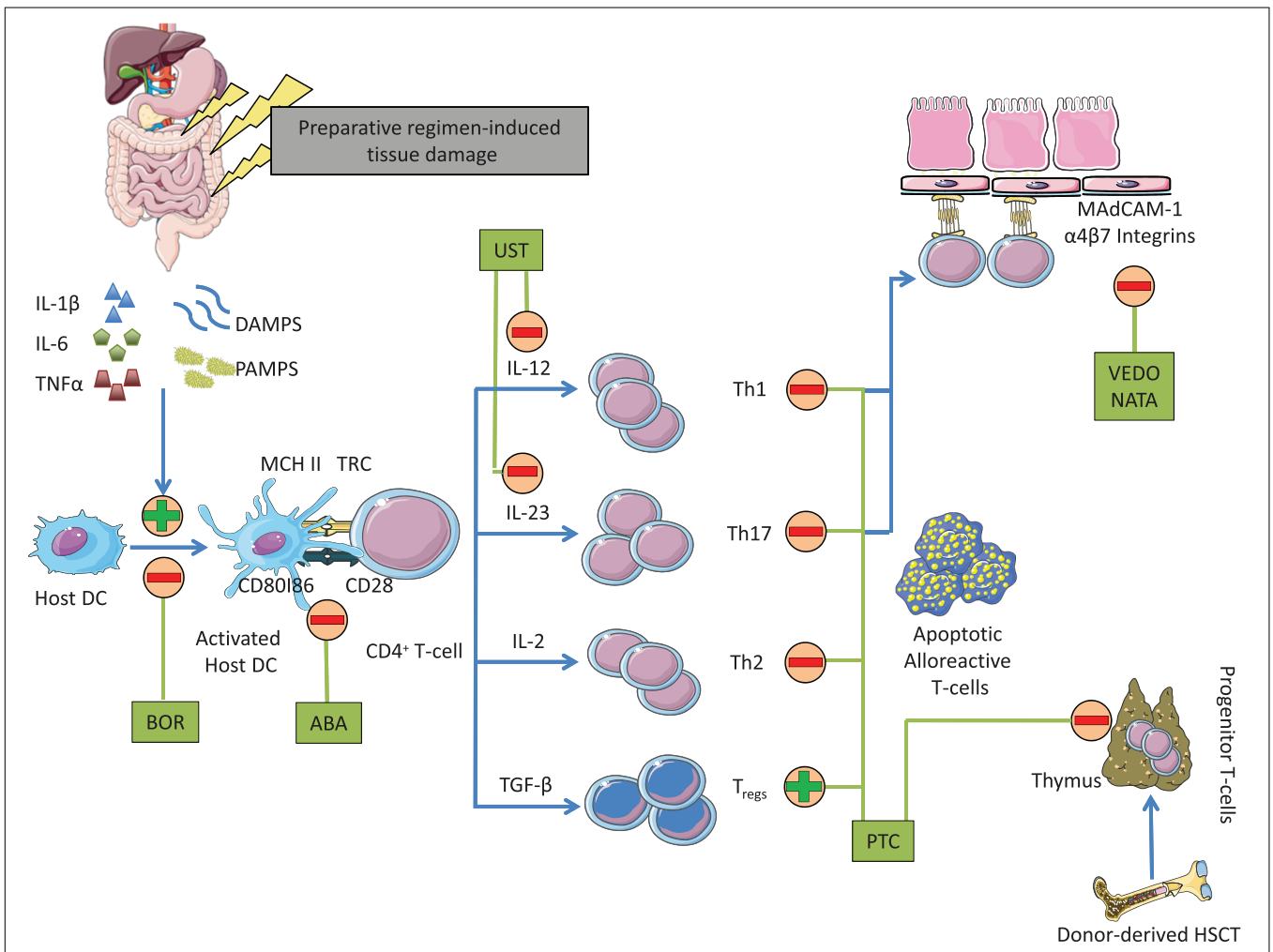

FIGURE 1 | Mechanism of action of agents that may conceptually be added to PTCy to develop CN and mTORI-free combinations for GvHD prevention. DAMPS, damage-associated molecular patterns; PAMPS, pathogen-associated molecular patterns; BOR, bortezomib; ABA, abatacept; MHC II, major histocompatibility complex class II; UST, ustekinumab; TGF-β, transforming growth factor-β; HSC, hematopoietic stem cells; VEDO, vedolizumab; NATA, natalizumab.

13% (90% CI 9–16%). The 1-year incidence of cGvHD was 28% (90% CI 20%–36%) and 28% (90% CI 33%–43%), respectively. The 1-year GRFS rates were also superior in the PTCy group (HR = 0.72, 95% CI 0.54–0.94, $p = 0.044$). However, there was no difference in TRM, DFS, and OS. These results were corroborated by a prospective randomized trial comparing CSA and MMF to PTCy and CSA following MRD and MUD peripheral blood transplants. The group receiving PTCy-based GvHD prophylaxis had lower rates of acute and cGvHD and improved GRFS (38).

In summary, pending the results of an ongoing phase III randomized trial (CTN03959241), comparing PTCy in combination with TAC and MMF to TAC and MTX, PTCy in combination with a CNI for GvHD prophylaxis may potentially emerge as a new standard of care for the prevention of GvHD in the setting of MRD and MUD transplantation.

PTCy AS A PLATFORM FOR CN AND mTOR INHIBITOR-FREE GvHD PREVENTION IN MRD AND MUD TRANSPLANT

Given the previously mentioned pitfalls of CN and mTOR inhibitor-containing GvHD prevention regimens, our work over the last several years focused on exploiting PTCy in order develop CN and mTOR inhibitor-free GvHD preventive combinations.

Proteasome inhibitors have multiple immune modulatory effects that span different stages of GvHD development including dendritic and T-cell differentiation, proliferation and function. Proteasome inhibitors also foster the expansion of regulatory T-cells (39, 40). Despite the fact that the results of a recent phase II trial examining the combination of bortezomib with CN and mTORI compared to a standard TAC and MTX combination were disappointing (41), we hypothesized based on pre-clinical data that proteasome inhibitors remain appealing agents when paired with PTCy. In a murine model, the combination of PTCy and ixazomib resulted in superior survival of animals subjected to lethal GvHD in comparison to either drug alone. Furthermore, PTCy prevented the surge in interleukin-1β (IL-1β) and donor T-cell expansion characteristic of delayed administration of proteasome inhibitors following transplantation. The combination induced profound post-transplant cytokine suppression including IL-6, IL-1β, and tumor necrosis factor-α (42). In a clinical trial, bortezomib was added to PTCy in MRD and MUD transplantation following RIC and peripheral blood grafts. Patients receiving MUD transplantation also received r-ATG. Two doses of bortezomib were given 6 h after graft infusion and 72 h thereafter. All GvHD prophylaxis was completed on day +4. The rates of aGvHD grades II to IV and III to IV were 35.9% (95% CI 18.6–53.6%) and 11.7% (95% CI 2.8%–27.5%). The rate of cGvHD was 27% (95% CI 11.4%–45.3%). The 2-year GRFS was 37.7% (95% CI 20.1%–55.3%) (43). When compared to a registry control group the 1-year GRFS was 39% (95% CI 24%–54%) in the study group and 32% (95% CI 27%–38%) in the control group (HR = 0.81, 90% CI 0.52–1.27, $p = 0.44$) (unpublished data). These promising results are being confirmed in a larger trial.

Table 1 provides a summary of selected registered studies that use PTCy-based GvHD prevention in MRD and MUD transplantation.

FUTURE DIRECTIONS

Improving upon our understanding of GvHD pathophysiology and our advancement in drug development offer additional opportunities to rationally design CN and mTORI-free PTCy-based GvHD prevention combinations following MRD and MUD transplantation. Toward this end, T-cell co-stimulation blockade, integrin antagonists and IL-23 inhibitors seem attractive as these agents target different phases of GvHD development (**Figure 1**). Abatacept, a soluble fusion compound of cytotoxic T-cell associated antigen-4 (CTLA-4) and immunoglobulin G1 (IgG1) that binds to CD80 and prevents dendritic cells from delivering a second stimulation signal for T-cell activation, reduced the incidence of aGvHD when added to a standard combination of CNI and MTX in patients receiving matched or one HLA locus mismatched-unrelated donor transplantation (44). Anti-integrin therapy, on the other hand, prevents T-cell trafficking into the guts. Vedolizumab, which acts on a gut-trophic α4β7 integrin and natalizumab, which acts on α4-integrin are being examined in GvHD prevention and treatment (45, 46). Lastly, IL-23 subunit p40 antagonist, ustekinumab, polarizes T-cell differentiation thus preventing the development of T-helper 1 (Th1) and T-helper 17 (Th17) and favoring the expansion of regulatory T-cells is also being studied in GvHD prevention (47). Other IL-23 p19 subunit inhibitors including guselkumab, tildrakizumab and risankizumab may also be of interest. Carefully designed clinical trials are warranted to examine the potential role of these agents in the prevention of GvHD.

CONCLUSION

Since establishing its role in HLA-haploidentical transplantation, PTCy has emerged as an effective platform in GvHD prevention strategies in MRD and MUD transplantation. Pending ongoing randomized study, PTCy in combination with TAC and MMF may represent a new standard of care based on its ease of administration and efficacy. Furthermore, PTCy offers a unique opportunity for the development of CN and mTORI-free GvHD preventive combinations, allowing an early introduction of immune manipulations and small molecules aimed to prevent disease relapse following allogeneic BMT.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript.

REFERENCES

1. Singh AK, McGuirk JP. Allogeneic stem cell transplantation: a historical and scientific overview. *Cancer Res.* (2016) 76:6445–51. doi: 10.1158/0008-5472.CAN-16-1311
2. Ram R, Gafter-Gvili PV, Yeshurun M, Paul M, Raanani P, Shpilberg O. Prophylaxis regimens for GvHD: systemic review and meta-analysis. *Bone Marr Transplant.* (2009) 43:643–53. doi: 10.1038/bmt.2008.373
3. Przepiorka D, Nash RA, Wingard JR, Zhu J, Maher RM, Fitzsimmons WE, et al. Relationship of tacrolimus whole blood levels to efficacy and safety outcomes after unrelated donor marrow transplantation. *Biol Blood Marrow Transplant.* (1999) 2:94–7.
4. Luznik L, O'Donnell PV, Fuchs EJ. Post-transplantation cyclophosphamide for tolerance induction in HLA-haploidentical BMT. *Semin Oncol.* (2012) 39:683–93. doi: 10.1053/j.seminoncol.2012.09.005
5. Luznik L, Bolaños-Meade J, Zahurak M, Chen AR, Smith BD, Brodsky R, et al. High-dose cyclophosphamide as single-agent, short course prophylaxis of graft-versus-host disease. *Blood.* (2010) 115:3224–30. doi: 10.1182/blood-2009-11-251595
6. Kanakry CG, O'Donnell PV, Furlong T, de Lima MJ, Wei W, Medeot M, et al. Multi-Institutional study of post-transplantation cyclophosphamide as a single-agent graft-versus-host disease prophylaxis after allogeneic bone marrow transplantation using myeloablative busulfan and fludarabine conditioning. *J Clin Oncol.* (2014) 32:3497–505. doi: 10.1200/JCO.2013.54.0625
7. El Fakih R, Hashmi S, Ciurea SO, Luznik L, Gale RP, AlJurf M. Post-transplant cyclophposphamide use in matched HLA donors: a review of literature and future application. *Bone Marr Transplant.* (2020) 55:40–7. doi: 10.1038/s41409-019-0547-8
8. Whirl-Carrillo M, McDonagh EM, Hebert JM, Gong L, Sangkuhl K, Thom CF, et al. Pharmacogenomics knowledge for personalized medicine. *Clin Pharmacol Therap.* (2012) 92:414–7. doi: 10.1038/clpt.2012.96
9. Emadi A, Jones RJ, Bodsky RA. Cyclophospahmide and cancer: golden anniversay. *Nat Rev Clin Oncol.* (2009) 6:638–47. doi: 10.1038/nrclinonc.2009.146
10. Strauss G, Osen W, Debatin KM. Induction of apoptosis and modulation of activation and effector function in T cells by immunosuppressive drugs. *Clin Exp Immunol.* (2002) 128:255–66. doi: 10.1046/j.1365-2249.2002.01777.x
11. Al-Homsi AS, Roy T, Cole K, Feng Y, Duffner U. Post-transplant high-dose cyclophosphamide for the prevention of graft-versus-host disease. *Biology of Blood and Marrow Transplant.* (2014) 21:604–11. doi: 10.1016/j.bbmt.2014.08.014
12. Mussetti A, Greco R, Peccatori J, Corradini P. Post-transplant cyclophosphamide, a promising anti-graft versus host disease prophylaxis: where do we stand? *Exp Rev Hemtol.* (2017) 10:479–92. doi: 10.1080/17474086.2017.1318054
13. Cieri N, Peccatori J, Oliveira G, Greco R, Marktel S, Lunghi F, et al. Tracking T cell dynamics in the first month after haploidentical HSCT with post-transplant cyclophosphamide reveals a predominant contribution of memory stem T cells to the early phase of immune reconstitution. *Blood.* (2013) 122:4615. doi: 10.1182/blood.v122.21.4615.4615
14. Eto M, Mayumi H, Tolmita Y, Yoshikai Y, Nishimura Y, Maeda T, et al. Specific destruction of host-reactive mature T cells of donor origin prevents graft-versus-host disease in cyclophosphamide-induced tolerant mice. *J Immunol.* (1991) 146:1402–9.
15. Huyan XH, Lin YP, Gao T, Chen RY, Fan YM. Immunosuppressive effect of cyclophosphamide on white blood cells and lymphocyte subpopulations from peripheral blood of Balb/c mice. *Int Immunopharmacol.* (2011) 11:1293–7. doi: 10.1016/j.intimp.2011.04.011
16. Kanarky C, Ganguly S, Zahurak M, Bolaños-Meade J, Thoburn C, Perkins B, et al. Aldehyde dehydrogenase expression drives human regulatory t cell resistance to posttransplant cyclophosphamide. *Sci Transl Med.* (2013) 5:211. doi: 10.1126/scitranslmed.3006960
17. Ganguly S, Ross DB, Panoskaltsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, et al. Donor-derived CD4+ Foxp3+ regulatory cells are necessary for post-transplantation cylophosphamide-mediated protection against GVHD in mice. *Blood.* (2014) 124:2131–41. doi: 10.1182/blood-2013-10-525873
18. Wachsmuth LP, Patterson M, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Posttransplantation cyclophosphamide prevents graft-versus-host disease by induced alloreactive T cell dysfunction and suppression. *J Clin Invest.* (2019) 129:2357–73. doi: 10.1172/JCI124218
19. Radjocic V, Luznik L. Mechanism of action of posttransplantation cyclophosphamide: more than meets the eye. *J Clin Inves.* (2019) 129:2189–91. doi: 10.1172/JCI128710
20. O'Donnell PV, Luznik L, Jones RJ, Vogelsang GB, Leffell MS, Phelps M, et al. Nonmyeloablative bone marrow tranaplantation from partially HLA-matched related donors using posttransplantation cyclophosphamide. *Biol Blood Marr Transplant.* (2002) 8:377–86. doi: 10.1182/bloodadvances.2016002766
21. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose posttransplantation cyclophosphamide. *Biol Blood Marr Transplant.* (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
22. Kasamon YL, Luznik L, Leffell MS, Kowalski J, Tsai HL, Bolaños-Meade J, et al. Nonmyeloablative HLA-haploidentical bone marrow transplantation with high-dose posttransplantation cyclophosphamide: effect of HLA disparity on outcome. *Biol Bood Marrow Transplant.* (2010) 16:482–9. doi: 10.1016/j.bbmt.2009.11.011
23. Munchel A, Kesserwan C, Symons HJ, Luznik L, Kasamon YL, Jones RJ, et al. Nonmyeloablative HLA-haploidentical bone marrow transplantation with high dose post-transplantation cyclophosphamide. *Pediatr Rep.* (2011) 3(Suppl. 2):43–7. doi: 10.4081/pr.2011.s2.e15
24. Solomon SR, Sizemore CA, Sanacore M, Zhang X, Brown S, Holland HK, et al. Haploidentical tranplantation using T-cell replete peripheral blod stem cells and myelaobaltive conditioning in patients with high risk hematologic malignancies who lack conventional donors is well tolerated and produces excellent relapse-free survival: results of prospective phase II trial. *Biol Blood Marr Transplant.* (2012) 18:1859–66. doi: 10.1016/j.bbmt.2012.06.019
25. Bhamidipati PK, Dipersio JF, Stokerl-Goldstein K, Rashidi A, Gao, F, Uy GL, et al. Haploidentical transplantation using G-CSF-mobilizaed T-cell replete PBSCs and post-transplant CY after non-myelablative conditioning regimen is safe and is assocaited with favorable outcomes. *Bone Marr Transplant.* (2014) 48:1124–26. doi: 10.1038/bmt.2014.108
26. Wang Y, Wu DP, Liu QF, Xu LP, Liu KY, Zhang XH, et al. Low-dose post-transplant cyclophophamide and anti-thymocyte globulin as an effective strategy for GVHD prevention in haploidentical patients. *J Hematol Oncol.* (2019) 12:88. doi: 10.1186/s13045-019-0781-y
27. McCurdy SR, Luznik L. How we perform haploidentical stem cell transplantation with posttransplant cyclophosphamide. *Blood.* (2019) 134:1802–10. doi: 10.1182/blood.2019001323
28. Kankary C, Tsai HL, Bolanos-Meade J, Douglas Smith B, Gojo I, Kankary JA, et al. Single-agent GVHD prophylaxis with posttransplantion cylophosphamide after myeloablative, HLA-matched BMT for AML, ALL, and MDS. *Blood.* (2014) 124:3817–27. doi: 10.1182/blood-2014-07-587477
29. Alousi A, Brammer JE, Saliba RM, Andersson B, Popat U, Hosing C, et al. *P*hase II trial of GVHD prophylaxis with post-transplanatation cyclophosphamide following reduced-intensity busulfan/fludarabine (Bu/Flu) conditioning for hematologic malignancies. *Biol Blood Marr Transplant.* (2015) 21:906–12. doi: 10.1016/j.bbmt.2015.01.026
30. Holtick U, Chemnitz J, Shimabukuro-Vornhagen A, Theurich S, Chakupurakal G, Krause A, et al. OCTET-CY: a phase II study to investiagte the efficacy of a post-transplant cyclophosphamide as sole graft-versus host prophylaxis after allogeneic peripheral blood stem cell transplantation. *Euro J Hematol.* (2016) 96:27–35. doi: 10.1111/ejh.12541
31. Bradstock KF, Bilmon I, Kwan J, Micklethwaite K, Blyth E, Deren S, et al. Single-agent high-dose cyclophosphamide for graft-versus-host disease prophylaxis in human leukocyte antigen-matched reduced-intensity peripheral blood stem cell transplantation. *Biol Blood Marr Transplant.* (2015) 21:934–53. doi: 10.1016/j.bbmt.2015.01.020
32. Mielcarek M, Furlong T, O'Donnell PV, Storer BE, McCune JS, Storb R, et al. Posttransplantation cyclophosphamide for prevention of graft-versus-host disease after HLA-matched mobilized blood cell transplantation. *Blood.* (2016) 127:1502–8. doi: 10.1182/blood-2015-10-672071

33. Moiseev IS, Pirogova OV, Alyanski AL, Babenko EV, Gindina TL, Darskaya EI, et al. Graft-versus-host disease prophylaxis in unrelated peripheral stem cell transplantation with post-transplantation cyclophosphamide, tacrolimus, and mycophenolate mofetil. *Biol Blood Marr Transplant.* (2016) 22:1037–42. doi: 10.1016/j.bbmt.2016.03.004
34. Carnevale-Schianca F, Caravelli D, Gallo S, Coha V, D'Ambrosio L, Vassallo E, et al. Post-Transplant cyclophosphamide and tacrolimus-mycophenolate mofetil combination prevents graft-versus-host disease in allogeneic peripheral blood hematopoietic cell transplantation from HLA-matched donors. *Biol Blood Marr Transplant.* (2017) 23:459–66. doi: 10.1016/j.bbmt.2016.12.636
35. Solomon SR, Sanacore M, Zhang X, Brown S, Holland K, Morris LE, et al. Calcineurin inhibitor-free graft-versus-host disease prophylaxis with post-transplantation cyclophosphamide and grief-course sirolimus following reduced-intensity peripheral blood stem cell transplantation. *Biol Blood Marr Transplant.* (2014) 20:1828–34. doi: 10.1016/j.bbmt.2014.07.020
36. Greco R, Lorentino F, Morelli M, Giglio F, Mannina D, Assanelli A, et al. Posttransplantation cyclophosphamide and sirolimus for prevention of GvHD after HLA-matched PBSC transplantation. *Blood.* (2016) 128:1528–31. doi: 10.1182/blood-2016-06-723205
37. Bolanos-Meade J, Reshef R, Fraser R, Fei M, Abhyankar S, Al-Kadhimi Z, et al. Three prophylaxis regimens (tacrolimus, mycophenolate mofetil, and cyclophosphamide; tacrolimus, methotrexate, and bortezomib; or tacrolimus, methotrexate, and maraviroc) versus tacrolimus and methotrexate for prevention of graft-versus-host disease with haemopoietic cell transplantation with reduced-intensity conditioning: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). *Lancet Haematol.* (2019) 6:e132–43. doi: 10.1016/S2352-3026(18)30221-7
38. De Jong CN, Meijer E, Bakunina K, Nur E, Kooji MVM, de Groot M, et al. Post-transplantation cyclophosphamide after allogeneic hematopoietic stem cell transplantation: results of the prospective randomized HOVO-96 trial in recipients of matched related and unrelated donors. *Blood.* (2019) 134(suppl. 1):1. doi: 10.1182/blood-2019-124659
39. Mohty M, Brissot E, Savani BN, Gaugler B. Effects of bortezomib on the immune system; a focus on immune regulation. *Biol Blood Marr Transplant.* (2013) 19:1416–20. doi: 10.1016/j.bbmt.2013.05.011
40. Al-Homsi AS, Feng Y, Duffner U, Al-Malki MM, Goodyke A, Cole K, et al. Borteaomib for the prevention and treatment of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Exp Hematol.* (2016) 44:771–7. doi: 10.1016/j.exphem.2016.05.005
41. Koreth J, Kim HT, Lange PB, Poryanda SJ, Reynolds CG, Rai SC, et al. Bortezomib-based immunosuppression after reduced-intensity conditioning hematopoietic stem cell transplantation; randomized phase II results. *Haematologica.* (2018) 103:522–30. doi: 10.3324/haematol.2017.176859
42. Al-Homsi AS, Goodyke A, McLAne M, Abdel-Mageed S, Cole K, Cole K, et al. Post-transplation cyclopopsphamide and ixazomib combination rescues mice subjected to experimental graft-versu-host disease and is superior to either agent alone. *Biol Blood Marr Transplant.* (2017) 23:255–61. doi: 10.1016/j.bbmt.2016.11.015
43. Al-Homsi AS, Cole K, Muilenburg M, Goodyke A, Abidi M, Duffner U, et al. Calcineurin and mTOR inhibitor-freep post-transplantation cyclophosphamide and bortezomib combination for graft-versus-host disease prevention after peripheral blood allogeneic hematopoietic stem cell transplantation: a phase I/II study. *Biol Blood Marr Transplant.* (2017) 23:1651–7. doi: 10.1016/j.bbmt.2017.05.024
44. Watkins BK, Qayed M, Bratrude B, Betz K, Sinclair S, Suessmuth Y, et al. T cell costimulation blockade with CTLA4-Ig (abatecept) for acute Gvhd prevention in HLA matched and mismatched unrelated donor transplantation: results of the first phase 2 trial. *Biol Blood Marr Transplant.* (2019) 25(Suppl. 3):S51–52. doi: 10.1016/j.bbmt.2018.12.129
45. Park SC, Jeen YT. Anti-integrin therapy for inflammatory bowel disease. *Worl J Gastroenterol.* (2018) 24:1868–80. doi: 10.3748/wjg.v24.i17.1868
46. FlØisand Y, Lundin KEA, Lazarevic V, Kristiansen JD, Osnes LTN, TjØnnfjord GE, et al. Targeting integrin α4β7 in steroid-refractory intesntinal graft-versus-host disease. *Biol Blood Marrow Transplant.* (2017) 23:172–5. doi: 10.1016/j.bbmt.2016.10.009
47. Pidala J, Beato F, Kim J, Betts B, Jim H, Sagatys E, et al. *In vivo* IL-12/IL-23p40 neutralization blocks Th1/Th17 response after allogeneic hematopoietic cell transplantation. *Heamtologica.* (2018) 103:531–9. doi: 10.3324/haematol.2017.171199

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